CHARACTERIZATION AND EVALUATION OF PAPAYAS GENETICALLY TRANSFORMED WITH THE COAT PROTEIN GENE OF PAPAYA RINGSPOT VIRUS

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ABSTRACT

Genetically engineered papayas (*Carica papaya* L.) transformed with the coat protein gene of papaya ringspot virus (PRV) were characterized for morphology, fertility, transgene expression and evaluated for viral disease resistance. Among the R_0 plants examined were 18 lines produced by means of particle bombardment and 4 lines produced by *Agrobacterium* transformation. Most of the plant lines appeared to be normal in morphology and fertility, except for a few that showed either a certain level of sterility or polyploidy.

Selected R_1 progenies resulting either from self-pollinations of R_0 plants or from crosses with non-transgenic plants, were evaluated in the greenhouse. Progeny analyses showed that most transgenes segregated in a simple Mendelian fashion. Progeny derived from R_0 line 55-1 showed a high level of protection against PRV infection, while other R_1 progenies showed variable responses, from delay of symptoms to complete susceptibility to the virus.

Two R_0 lines and three of the coat protein positive (CP+) R_1 lines were evaluated in the field for resistance to PRV. CP+ R_0 line 55-1 and its subsequent R_1 progeny showed a very good level of resistance to PRV, while other lines tested were either susceptible or showed only delayed symptoms following inoculation by the virus. The resistant

line 55-1 showed vigorous growth with normal morphology and acceptable fruit quality under field conditions.

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INTRODUCTION

Papaya is one of the most popular breakfast and dessert fruits in the tropics, and serves also as a vegetable staple in some Southeast Asian countries. It is commonly grown in gardens and dooryards in tropical lowland regions because of its palatability and continuous bearing habit throughout the year. Papayas also provide an important dietary source of vitamin A and C.

Several diseases, including those caused by fungi and nematodes, have posed problems in papaya production. However, in most parts of the world, papaya production has been limited primarily due to susceptibility to papaya ringspot virus (PRV) disease. There has been little or no resistant germplasm good enough for conventional breeding purposes, while other control measures, such as sanitation, quarantine and cross protection, have provided only a partial and temporary solution to the problem.

Hawaii is one of the major papaya producers in the world. PRV is found in some areas of Hawaii, but not in others. However, the disease pressure has been increasing since it was first found on Oahu island in 1945, and has recently posed a threat to Hawaii's main production area in the Puna district on the island of Hawaii. Since 1992, many plants have been destroyed in an attempt to halt the spread of the virus, without success.

Papaya ringspot virus has no chemical cure. With no resistant cultivar available at hand, cross protection has offered a partial solution to the matter. Despite some economic benefits to be gained with cross protection, this measure suffers from several drawbacks. Most importantly, there is a 15 to 20% crop loss associated with the mild strain virus infection, which may still be acceptable under high disease pressure situations.

Genetic engineering provides a quick way to address disease problems in a crop that might otherwise take years to achieve through classical breeding strategies. Genetically engineered papaya with a high level of resistance to Hawaiian strain of PRV have been created by transforming Hawaiian papaya cultivars with the coat protein (CP) gene of the mutant mild virus strain PRV HA 5-1 (Fitch 1991, Fitch et al. 1990, 1992, 1993). These plants are currently being characterized and evaluated under greenhouse and field conditions to determine the utility of the coat protein mediated resistance to PRV. This thesis work will include study of the original R_0 transgenic papaya plants, as well as their R_1 sexual progenies. The objectives of this study are 1) to characterize the 22 R_0 transgenic papaya lines with respect to fertility, fruit quality, occurrence of somaclonal mutations, and expression of the transgenes (NPTII, GUS, and CP), 2) to determine the expression and inheritance of transgenes in selected R_1 progenies, and 3)

to assess expression and inheritance of PRV resistance in selected R_0 plants and R_1 progenies under greenhouse and field conditions.

LITERATURE REVIEW

Papaya (*Carica papaya* L.) is a polygamous species belonging to the dicotyledonous family Caricaceae, which consists of four genera: *Carica*, *Cylicomorpha*, *Jacaratia*, and *Jarilla* (Badillo, 1971). *Carica* is the largest genus, consisting of about 22 species native to tropical America with chromosome numbers of 2n=18.

Papaya is one of the most widely cultivated and economically valuable fresh fruit crops in the tropics and subtropics. The 1990 FAO Production Yearbook reported papaya production as 3.9 million metric tons in 1989, with most production in the developing countries. In Hawaii, papaya yields about $16 million and ranks as the fourth most valuable agricultural commodity after sugarcane, pineapple, and macadamia nuts.

Papaya is a fast growing semi-woody tree, usually unbranched, with a hollow trunk 10 to 30 cm in diameter and up to 10 m in height (Purseglove 1968). Plants can live 25 years or more (Storey 1953), but commercial plantation keeps the plants in production for only 3 to 4 years, by which time the plants are already too tall to harvest and may have developed serious root disease problems.

Papaya is utilized as a popular dessert fruit in many countries and as a vegetable staple in some Southeast Asian areas. The fruit also is processed for making soft drinks,

jam, ice cream flavoring, and canned slices in syrup. Since the latex contains the proteolytic enzyme papain, green fruits are often cooked with meat to act as a tenderizer. Papain extracted from immature fruits is also used in a variety of ways, such as in the manufacture of chewing gums, in cosmetics, and as a drug for digestive ailment. Leaves and flowers are also consumed as vegetables by Southeast Asians. In some countries, seeds are uses as a vermifuge and abortifacient (Purseglove 1968, Storey 1976).

Studies of the genetics of papaya have revealed that it has three primary sex types: male, hermaphrodite, and female; these phenotypes are controlled by three alleles at a single locus (Storey 1953). The sex of individual plants can be determined only after floral anthesis, which commences about 4 to 6 months after germination. Sex expression and fruit production in male and hermaphrodite plants are subject to modification by seasonal and environmental factors (Storey 1976, Arkle and Nakasone 1984). Flesh color of the fruit ranges from yellow to red and is determined by a single major gene, at which locus the allele for yellow flesh is dominant to red (Storey 1969). The fruit is rich in vitamin A and C, and its flavor is best when the fruit is fully ripe.

Papaya was first introduced to Hawaii in the early 1800s, but only after the introduction of the "solo" type of papaya from Barbados and Jamaica in 1911 has papaya industry

in Hawaii changed its outlook. Solo papayas are gynodioecious papayas with sweet fruits which are small enough to be consumed by one person.

Papaya in Hawaii has several disease problems, such as root rot diseases caused by the fungi *Phytophthora* and *Pythium* (Mosqueda-Vazquez et al. 1981), anthracnose caused by *Colletotrichum gloeosporioides* (Alvarez and Nishijima 1987), powdery mildew caused by *Oidium caricae*, and nematode diseases caused by *Meloidogyne* spp. and *Rotylenchulus reniformis* (Hine et al. 1965). But none of these diseases is as serious as the disease caused by papaya ringspot virus (PRV) in posing a threat to the papaya industry in Hawaii, as well as in other parts of the world. The name PRV was first coined in 1940s to describe the virus disease in Hawaii (Jensen 1949) and it has also been one of the most destructive disease for papaya production in Brazil, the Caribbean, Mexico, USA (Florida and Hawaii), Australia, Africa, the Philippines, Taiwan, Thailand and China (Purcifull et al. 1984, Yeh et al. 1988).

PRV is a member of Potyvirus genus, belonging to the plant Potyviridae family (Martelli, 1992). The virus has flexuous filamentous particles measuring about 780 X 12 nm. It has a positive sense, single-stranded RNA genome and is transmitted non-persistently by aphids (Purcifull et al. 1984). Three major strains of PRV have been identified: PRV-P which causes the disease problem in papaya, PRV-W which

can not infect papaya, but causes major diseases in cucurbits, and PRV-T which is also non-pathogenic on papaya, but has similar biological properties as PRV-W (Quiot-Douine et al. 1986, 1990). In this work, PRV-P is the pathogen we are interested in, and for convenience will be referred to as PRV throughout the rest of this thesis. The virus has been studied extensively and its complete nucleotide sequence has been revealed (Yeh et al. 1992) and compared with other strains (Quemada et al. 1990, Bateson and Dale 1991, Wang and Yeh 1992).

PRV infects plants in only three families: Caricaceae, Chenopodiaceae, and Cucurbitaceae (Purcifull et al. 1984, Yeh et al. 1984). In papaya, the disease causes symptoms of mottling and distortion on leaves, rings and spots on fruit which are more prominent in immature fruit, and greasy lesion streaks on stems and petioles. The plants are stunted, set less fruits and of poor quality, and eventually will die. Disease symptoms usually are more pronounced during cool weather and alleviate in warmer weather (Nishina et al. 1989). Plants infected with PRV have been shown to have reduced photosynthetic efficiency and increased dark respiration (Marler et al. 1993). There is no chemical cure to the virus, and once established in an area, it poses a serious problem to papaya production, since it is very difficult to eradicate.

There are several measures that have been used to

control PRV disease. Quarantine regulations to restrict plant movement are useful in excluding PRV from geographically isolated production areas, but once the virus has been introduced, roguing of diseased plants and insecticide applications against insect vectors are not effective to control the disease. Classical breeding to develop resistant cultivars has been tried with no great success, since no cultivar with good resistance is available. Tolerant varieties are available, but apparently the trait is inherited in a quantitative manner (Conover and Litz 1978), which is difficult to use for breeding purposes. A tolerant dioecious line originating from Colombia has been used in Florida to produce the tolerant dioecious cultivar 'Cariflora' (Conover and Litz 1978, Conover et al. 1986); and in Hawaii (Zee 1985), however, no complete resistance was obtained. A breeding program is currently being conducted at the University of Hawaii to select tolerant gynodioecious lines from crosses between solo cultivars and the PRV-tolerant Colombian germplasm. Analyses of the progeny are currently under way (Manshardt, personal communication). Interspecific hybridization with resistant wild *Carica* species has been achieved with great difficulty due to reproductive barriers (Manshardt and Wenslaff 1989a, 1989b). The resulting hybrids were quite sterile, and backcrosses have proven to be difficult.

A lack of resistant papaya cultivars and the restricted

host range of PRV make cross protection an attractive strategy for control of PRV disease. Cross protection, in which the deliberate inoculation of a crop with a mild strain of a virus gives protection against economic loss by subsequent infection by a severe strain of the same virus, has been tried in some parts of the world with a certain degree of success (Yeh and Gonsalves 1984, Wang et al. 1987, Yeh et al. 1988, Gonsalves 1989). However, cross protection has some major drawbacks which might restrict its application (Fulton 1986, Ponz and Bruening 1986). The protected plants are not immune, and will even produce symptoms due to infection by the mild strain. Superinfection occurs under high disease pressure of the challenge virus (Wang et al. 1987). There is concern that the mild virus might escape to other crops, or produce a synergistic reaction with an unrelated virus to exacerbates disease expression in a crop. The mild strain may also be subject to reverse mutation, causing it to assume wild type characteristics. Finally, the cost and difficulty involved with pre-inoculating each papaya generation before planting, and the associated 15 to 20% yield loss due to the protecting virus, make growers hesitate to practice this control measure.

Several models have been proposed for the mechanism of cross protection. The most commonly cited ones are: 1) the coat protein of the protecting virus can reencapsidate and

prevent uncoating of the challenge virus (DeZoeten and Fulton 1975, Wilson 1985, Wilson and Watkins 1986), 2) RNA-RNA hybridization between the RNAs of the two virus strains inhibits replication of the challenge strain (Palukaitis and Zaitlin 1984).

Recent advances in genetic engineering have made gene transfer from unrelated organisms to a target crop possible. Sanford and Johnston (1985) proposed a general strategy for genetically engineering resistance to pathogens. When a host lacks resistance to a pathogen, or when such resistance is polygenic and difficult to transfer through conventional breeding methods, it may be practical to consider introducing a gene from the particular pathogen into the host, thus conferring resistance. If a key gene product of a pathogen is present in a dysfunctional form, in excess, or at the wrong developmental stage, it may disrupt the functionality of the pathogen while having little effect on the host. Any engineered viral gene in a plant, either expressing an encoded protein or not, that can interfere with a particular aspect of the viral life cycle may confer plant resistance to the virus. Thus, disruption of any stage of the viral life cycle, such as uncoating, translation, replication, or intercellular movement, can be a target for genetic engineering purposes. The pioneering work by Powell-Abel et al. (1986) has proved this concept of pathogen-derived resistance by transforming tobacco with tobacco

mosaic virus (TMV) coat protein gene. Since this first demonstration, there have been many other efforts to control virus diseases with pathogen-derived resistance, with varying degrees of success (Grumet 1990, Scholthof et al. 1993). Strategies other than the coat protein mediated protection have been utilized, including using complete viral genomes, antisense sequences, replicase sequences, movement protein sequences, satellite RNAs, defective interfering RNAs and pathogenesis related proteins (Grumet 1990, Scholthof et al. 1993). However, coat protein mediated protection is by far the most frequently adopted approach.

Genetic engineering for virus resistance through coat protein (CP) mediated protection has been applied in 12 different virus groups (Beachy 1993), including the control of papaya ringspot virus in papaya (Fitch 1991, Fitch et al. 1990, 1992, 1993). Transgenic plants expressing the coat protein, in general, showed delayed symptom expression, reduced symptom severity with fewer sites of infection on inoculated leaves, reduced systemic spread of the virus, lower virus concentrations in infected plants (Beachy et al. 1990). The CP protection may be overcome by inoculation with high concentration of virus, and it is mostly, but not always, susceptible to RNA infection (Hemenway et al. 1988, Okuno et al. 1993). Since protection by coat protein gene expression in transgenic plants mimics several characteristics of classical cross protection, the term

"genetically engineered cross protection" was applied (Nelson et al. 1987). In most cases, CP protection is relatively narrow, being effective only against strains closely related to the viral source of the CP gene expressed in the host (Beachy 1993). However, in some cases, there is evidence that the CP protection offers a broad spectrum resistance against different strains of the same virus or even against different viruses (Stark and Beachy 1989, Anderson et al. 1989, Ling et al. 1991, Namba et al. 1991, Quemada et al. 1991). Transgenic plants with high expression of CP usually exhibited high levels of protection against virus inoculation (Powell et al. 1990), although in certain cases, the level of CP expression did not correlate well with the level of protection (Stark and Beachy 1989, Namba et al. 1991, Quemada et al. 1991, Pang et al. 1992).

Mechanisms for CP resistance have been proposed (Beachy et al. 1990, Hanley-Bowdoin and Hemenway 1992). CP may interfere with an early event in viral infection, such as inhibiting the uncoating of the virus particle (Register and Beachy 1988, Wu et al. 1990), as suggested earlier by Wilson and Watkins (1986). Further, CP expression may prevent long distance systemic spread of the virus (Wisniewski et al. 1990, Lindbo and Dougherty 1992a, Okuno et al. 1993). Lindbo and Dougherty (1992a) reported that plants expressing truncated CP were even more effective in preventing intercellular virus movement than plants expressing the full

length version of CP. It has been shown that in most cases the CP per se, and not CP-RNA, is responsible for the resistance (Powell et al. 1990). But in some cases, plants without any detectable CP also showed resistance to viral infection (Pang et al. 1992), suggesting that CP transcript was responsible for the protection through a different mechanism. Similarly, Lindbo and Dougherty (1992a, 1992b) showed that plants expressing untranslatable CP sequences suppressed viral replication and were more resistant than plants expressing normal CP.

Transgenic plants expressing the antisense constructs have also been tried in several cases. Antisense RNAs hybridize with viral RNAs to form double-stranded RNAs. The RNA-RNA hybrids may be rapidly degraded in the nucleus, thereby blocking transport of viral RNAs to cytoplasm and inhibiting RNA translation. They may also interfere with the viral uncoating and replication processes (Rezaian et al. 1988, Powell et al. 1989, Nelson et al. 1993). However, in most cases, antisense constructs did not provide as good protection against virus infection as the CP (Cuozzo et al. 1988, Hemenway et al. 1988, Rezaian et al. 1988, Powell et al. 1989), although some had reported the effectiveness of antisense construct against virus infection (Nelson et al. 1993).

Expression of a non-structural replicase gene has been shown to provide high level resistance against TMV infection

in transgenic tobacco. The potential mechanism may involve a reduced rate of viral replication in the transgenic plants. Golemboski et al. (1990) found that transgenic plants expressing the 54 kDa putative replicase gene of tobacco mosaic virus were completely resistant to the same virus or RNA infection, though the expected protein was not detected in the transgenic plants. However, the resistance was shown to be strain specific. In contrast, plants expressing the complete 126 kDa replicase gene were as susceptible to infection as untransformed plants. Anderson et al. (1992) showed that expression of a truncated replicase gene from cucumber mosaic virus in transgenic tobacco plants resulted in high levels of resistance to the virus and RNA infection. Chlorotic lesions were found on inoculated leaves, but no systemic infections were observed, suggesting that inhibition of viral movement or replication at infection sites might account for the resistance. Donson et al. (1993) reported a broad resistance to different strains of tobamovirus in transgenic tobacco, conferred by the expression of a modified 183 kDa replicase gene of tobacco mosaic virus. However the resistance was not effective against cucumber mosaic virus, a virus from unrelated cucumovirus group. The lack of systemic infection by different tobamovirus indicated the protection was due to reduced multiplication in inoculated leaves.

Transgenic plants expressing dysfunctional movement

protein (MP) has been shown to have a certain level of resistance to TMV (Lapidot et al. 1993, Malyshenko et al. 1993) by interfering with the virus cell to cell movement. The transgenic plants showed a delay and less severe symptom expression compared with the control plants. MP modifies the function of plant plasmodesmata and facilitates virus movement (Deom et al. 1992). The inactive MP might pre-occupy target sites in plant cells, or compete with wild-type MP of the challenging virus. As the result, the virus spread will be reduced, and disease symptoms will be delayed or less severe. However, this strategy does not confer an absolute protection against TMV. Once sufficient wild-type MP accumulates during virus infection, the virus will be able to move to adjacent cells (Lapidot et al. 1993).

The use of satellite RNA (Sat-RNA) sequences for production of virus resistant transgenic plants has been successful in some cases. Sat-RNAs are single-stranded RNAs that are unable to multiply in host cells without the presence of a specific helper virus. However, they are not necessary for the helper virus multiplication and have no apparent sequence homology with the helper virus genome (Tien and Wu 1991). Some applications of Sat-RNAs produced good protection of crops against virus infections (Montasser et al. 1991, Gallitelli et al. 1991, Tien and Wu 1991). The protection mechanism involved replication competition, in which Sat-RNAs interfere or compete with viral genomic RNA

for replication. Transgenic plants that expressed Sat-RNA have been shown to be highly resistant to virus infection, independent of concentration of the inoculum (Harrison et al. 1987). A combination of CP and Sat-RNA expression in transgenic plants proved to be twice as effective as the CP gene or Sat-RNA alone (Yie et al. 1992). The enhancement of multi-trait protection was due to resistance provided by the CP gene during early stage of infection and by Sat-RNA at a later stage in viral replication. However, protection by the use of Sat-RNA sequences in transgenic plants has several limitations. Only a few viruses are known to have Sat-RNAs, and in many cases, the presence of Sat-RNAs can make symptoms even worse. Furthermore, they are subject to high mutation rates due to lack of proofreading during viral replication (Grumet 1990), and this could result in a loss of protection or an increase in symptom severity.

The use of defective interfering RNA (DI RNA) mediated protection in transgenic plants has been reported (Kollàr et al. 1993). DI RNAs are naturally occurring, truncated mutants of infectious viral genomic RNAs, which reduce the replication rate of the helper virus and cause milder virus symptoms in plants and animals. DI RNAs compete with the helper viral RNAs during replication and inhibit symptom expression caused by the helper virus (Roux et al. 1991). Transgenic plants accumulating the DI RNAs showed as high a level of resistance against virus and RNA inoculation as

those provided by naturally occurring DI RNAs (Kollár et al. 1993). However, the major drawback of this approach is that natural DI RNAs are not commonly present in plant viruses, though artificial DI RNAs were shown to act like the wild type ones (Marsh et al. 1991).

There are several other strategies that might be used for plant protection. Expression of the whole genome of a mild strain virus was shown to confer resistance to infection by a severe strain of the same virus, a phenomenon known from classical cross protection (Yamaya et at. 1988). Expression of pathogenesis-related host proteins which may be toxic to invading viruses, could be enhanced in transgenic plants to give protection against viral diseases (Grumet 1990). Small catalytic RNAs (ribozymes) which occur naturally in some virus systems, or the production of antibodies to specific viral proteins, could be engineered into plants for viral protection (Scholthof 1993).

The release of transgenic plants for agriculture use has been subject to regulations and risk assessment procedures. Conventional breeding methods are limited to the gene pool available for a crop species with its sexually compatible relatives. However, since it is now possible through the advance of biotechnology to introduce foreign genes that are inaccessible through traditional breeding, transferring into plants novel characteristics of which we have little knowledge or experience, there is a risk of

creating organisms that might negatively impact agriculture and local environments. Potential ecological risks that transgenic plants might present to the environment include primarily: 1) that the transgenic plants will become a new weed, 2) that the transgene will escape by hybridization with wild relatives, thus enhancing weediness in wild species and contaminating germplasm resources, 3) that there will be adverse effects in ecosystems by creating new biotypes of pathogens that overcome resistance in transgenic plants, and 4) the direct hazard to human, domestic animals or beneficial organisms (Tiedje et al. 1989, Crawley et al. 1993). Hence, researchers, government agencies and society generally agree about the need for regulation of the novel products of biotechnology (Tiedje et al. 1989, Dale 1992, 1993). Therefore, field testing of transgenic plants in carefully designed experiment with proper planning and regulatory oversight has been a mandatory step to establish the safety of the product under development (Wrubel et al. 1992, Tiedje et al. 1989). There have been more than 370 permits issued in 35 states since the US Department of Agriculture started regulating transgenic field trials (Kareiva 1993) and over 17 different crops have been tested worldwide (Dale 1993). To date, none of these has been approved by all regulatory agencies for commercial distribution.

Concerns of the USDA about transgenic plants center

mostly around the danger of weediness of the transformed plants in the field. Crawley et al. (1993) conducted one of the most comprehensive trials on transgenic oilseed rape under diverse growing environments, and concluded that there was no sign of more invasiveness in the transgenic plants, rather in some cases, the transgenic plants were less persistent than the untransformed counterparts. Keeler (1989) discussed the possibility of engineered crops becoming weeds, and concluded that average crops have to acquire several traits simultaneously should they become weeds, which is highly improbable for most crops. Moreover, transgenic plants are often assumed to be less fit than normal plants due to metabolic load in synthesizing new nucleic acids and proteins (Tiedje et al. 1989). However, analyses for weediness should be performed case by case, since some crops also have inherent weedy traits. Several preventive measures are recommended to reduce the risk of transgene escape from field trial (Ellstrand and Hoffman 1990, Wrubel et al. 1992). Perfect isolation of the field trial from local compatible relatives would be the best solution. However, determining isolation distance is not a clear cut matter. Most isolation distances have been rough estimates, sometimes based only on observation of accidental mating in the field, rather than on experimental evidence (Wrubel et al. 1992). Most self-fertilized crops require about 200 meter isolation distance, while outcrossing crops

require 1000 meter or more. Border rows consisting of a different species or the same species may act as a buffer to trap pollen entering or leaving the field trial. It is also necessary to put bags over male flowers to prevent pollen escape from the transgenic plants. Finally, there is a need to dispose of the transgenic plants or seeds after trial. Disking, burying, fumigating, herbicide spraying, autoclaving the plant materials have been used for the purpose.

MATERIALS AND METHODS

SOURCE OF TRANSGENIC PLANT MATERIALS

Transgenic papaya plants were produced in previous research using microprojectile bombardment and *Agrobacterium* transformation methods (Fitch 1991, 1993, Fitch and Manshardt 1990, Fitch et al. 1990, 1992, 1993). Three kinds of tissues were used, 90 to 105 days post-pollination immature zygotic embryos, hypocotyl sections from 14 days old seedlings, and embryonic callus and somatic embryos derived from both tissues. Two Hawaiian cultivars, 'Kapoho' and 'Sunset', were used for the transformation. Plasmids used were derivatives of the *Agrobacterium* binary vector pGA482 (An 1986). Plasmid pGA482GG (17 kb) containing the GUS and NPTII genes, and plasmids pGA482GG/cpPRV-4 and pGA482GG/cpPRV-19-5 (both 18.6 kb) containing, in addition, chimeric genes for the coat protein of the mild mutant PRV strain HA 5-1, were used for transformation. Thirteen 'Sunset' and 9 'Kapoho' regenerated R_0 lines (Table 1) were planted in the greenhouse at the University of Hawaii Magoon Facility and self-pollinated or crossed with pollen of non-transformed 'Kapoho', 'Sunset', 'Waimanalo' or 'Saipan Red' cultivars to obtain the R_1 generation.

Table 1. R_0 transgenic papaya lines grown in the greenhouse at Magoon Facility from 1991 to 1994

Line	Cultivar	Tissue origin	Type of transform.	Sex	No. of plants
19-1	K	SE	MB	F	4
39-1	K	ZE	MB	H	3
39-3	K	ZE	MB	H	2
39-4	K	ZE	MB	H	1
44-1	K	ZE	MB	H	1
AISAI-ID 1-132	K	SE	AT	H	1
AISAI-ID 1-137	K	SE	AT	F	1
AISAI-ID 4-2	K	SE	AT	H	1
AISAI-ID 4-4	K	SE	AT	H	1
33-2	SS	SE	MB	H	1
46-1	SS	ZE	MB	F	1
49-2	SS	ZE	MB	F	2
50-2	SS	ZE	MB	H	1
54-1	SS	ZE	MB	H	1
55-1	SS	ZE	MB	F	11
57-1	SS	ZE	MB	H	1
60-3	SS	ZE	MB	H	6
60-4	SS	ZE	MB	H	1
62-1	SS	ZE	MB	F	13
62-2	SS	ZE	MB	F	1
62-5	SS	ZE	MB	H	1
63-1	SS	ZE	MB	H	1

SE = culture originated from somatic embryo culture
ZE = culture originated from immature zygotic embryo
AT = *Agrobacterium* transformation
MB = microprojectile bombardment
F = female tree
H = hermaphrodite tree
K = 'Kapoho'
SS = 'Sunset'

CHARACTERIZATION OF R_0 TRANSGENIC PAPAYA PLANTS

Transgenic R_0 plants regenerated from tissue culture were planted in 25-gallon plastic pots, filled with potting mixture containing vermiculite, perlite and peat moss in 5:4:3 ratio. Three hundred grams of 14:14:14 Osmocote fertilizer and 250 grams of dolomite were added per 24 gallons of the potting material. One set of R_0 plants were planted in the summer of 1991, another set were planted in fall 1992. Plants were examined for growth and morpholgy to see whether abnormalities due to transformation or tissue culture had occurred. After anthesis, plants were pollinated to produce R_1 seeds. Hermaphrodite plants were self-pollinated by bagging the flower in a paper bag after either pollinating it with anthers from the same tree or gently tapping the flower to disperse the self pollen. Female plants were crossed with pollen from 'Kapoho', 'Sunset', 'Waimanalo' or 'Saipan Red' cultivars, and in some cases with pollen from transgenic hermaphrodite plants in the greenhouse. Flowers were tagged with the parental information and date of pollination. About 4 to 6 months later, ripe fruits were harvested and evaluated for quality by measuring the percentage of total soluble solids (TSS) with a refractometer. Seeds were also collected.

Plant fertility was assessed both for female and hermaphrodite trees. For hermaphrodite trees, anthers from

flowers at anthesis were placed on a glass microscope slide and squashed in a drop of acetocarmine stain to release the pollen grains. Pollen grains were stained for a few minutes, and observed under microscope. At least 3 flowers per tree, and 80 to 200 pollen grains per flower were examined each time. The percentage of darkly stained pollen grains was averaged for a particular tree, and in some cases, an average of several trees gave the fertility estimate of that particular line. In the case of female trees, relative fertility was assessed from the number of viable seeds produced.

Transgenic R_0 plants were also assayed for expression of the transgenes, including the reporter marker β-glucuronidase (GUS), selectable marker neomycin phosphotransferase II (NPTII) and coat protein (CP). These data extend the characterizations done previously by Fitch (1991).

GUS ASSAY

Histochemical staining using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) substrate was employed to assay GUS expression (Jefferson 1987). X-gluc was dissolved in 1/10 (weight/volume) dilution in dimethyl formamide, and diluted to a final concentration of 0.96 mM in 0.1 M sodium phosphate buffer pH 7.0. The second youngest, fully expanded leaf was sliced into 1.5 X 1.5 mm pieces, and 6 to 10 pieces

were put into the well of a polystyrene microtiter plate filled with 100 μl X-gluc solution. In some cases, R_1 papaya seeds were assayed by removing the seed coats and immersing the halved embryo/endosperm tissues in the X-gluc solution. A blue precipitate, resulting from oxidative dimerization of indolyl derivative, was observed on tissues which express GUS activity after incubation at 37°C for 1 to 10 hours. The leaf tissues were washed several times with 95% ethanol to extract chlorophyll pigmentation and enhance visualization of the blue precipitate, especially for tissues with weak GUS activity.

NPTII ASSAY

NPTII activity in transgenic tissue were assayed according to the protocol provided by 5 Prime - 3 Prime, Inc. (Boulder, CO) for its NPTII ELISA kit product, with slight modifications. Microtiter plate was coated with 1/1300 dilution of coating antibody in the buffer provided by the manufacturer, by pipetting 100 μl of solution into each well. Plate was incubated for 2 hours at 37°C or overnight at 4°C. The coating solution was then decanted and the wells were washed five times with PBST buffer (8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g NaN_3, per liter of aqueous solution, adjusted to pH 7.4, then add 0.5 ml Tween-20) and blotted dry on paper towels. After the wash, 200 μl of blocking buffer provided by the manufacturer

was applied to each well and the plate was incubated for 30 minutes at room temperature. The plate was then washed five times with PBST buffer and 100 μl of plant tissue sample was loaded into each well. Tissue samples were prepared by grinding the second youngest, fully expanded leaf in PBST-PVP buffer (PBST buffer supplemented with 2% polyvinyl pyrrolidone PVP-40) at 1/1250 (weight/volume) dilution. The plate was incubated either 2 hours at room temperature or overnight at 4°C. Following incubation, the plate was washed again with PBST buffer and 100 μl of biotinylated antibody at 1/1300 dilution in blocking buffer was loaded into each well. The plate was incubated for 1 hour at room temperature. Then the plate was washed with PBST buffer, and 100 μl of streptavidin conjugated alkaline phosphatase at 1/1000 dilution in blocking buffer was added to each well. The plate was incubated for 30 minutes at room temperature and washed with PBST buffer. Then 100 μl of *p*-nitrophenyl phosphate substrate at 2 mg/ml in substrate buffer (97 ml diethanolamine in 1 liter aqueous solution, adjusted to pH 9.8) was loaded into each well, and the plate was incubated for 30 to 40 minutes at 37°C. Color development was measured by absorbance at 405 nm wavelength (A_{405nm}) with a BioRad Model 450 microplate reader. The absorbance was compared with the reading from NPTII enzyme standards to determine the concentration of NPTII enzyme present in the leaf tissue.

To learn the assay sensitivity, the protein concentration of the leaf tissue was determined by Lowry method (Lowry et al. 1951) prior to NPTII assay. The leaf sample was ground at 1/5 dilution in 0.25 M Tris pH 7.8 containing 1 mM phenylmethylsulfonylfluoride (PMSF) using a small pestle in a 1.5-ml Eppendorf tube. The extract was spun at 12000 rpm for 30 minutes at 4°C, and water was added to 5 or 10 μl of supernatant to make a final sample volume of 0.2 ml. One ml of Folin C solution was added to the sample, mixed with a vortex mixer, and allowed to stand for 10 minutes at room temperature. Then 0.1 ml Folin E solution (1 N Folin-Ciocalteu's reagent) was added to the sample, and mixed immediately. The mixture was allowed to stand for 30 minutes at room temperature. Following incubation, the absorbance at 750 nm wavelength was recorded and translated to protein concentration by referring to a standard curve determined from bovine serum albumin (BSA) standard solutions.

COAT PROTEIN ASSAY

Expression of the CP gene was examined by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977). Monoclonal antibody against PRV HA 5-1, provided by Dr. D. Gonsalves (Department of Plant Pathology, Cornell University) was used to detect the CP since previous assays with polyclonal

antibody failed to detect transgenic CP expression (Fitch 1991). Each well of a microtiter plate was coated with 100 µl of 1 µg/ml antibody in coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1 liter aqueous solution, adjusted to pH 9.6) and incubated overnight at 4°C. The plate was washed three times with PBST buffer, with a 3-minute incubation within each wash, and blotted dry on paper towels. The second youngest, fully expanded leaf was ground at 1/60 (weight/volume) dilution in extraction buffer (200 ml of 0.25 M K_2HPO_4 and 0.1 M EDTA solution was mixed with 190 ml of 0.25 M KH_2PO_4 and 0.1 M EDTA solution to make pH 7.5 buffer), and 100 µl was loaded into each well. The plate was incubated overnight at 4°C and then washed 3 times as before with PBST buffer. The wells were then loaded with 100 µl of 1/1000 dilution of monoclonal antibody-enzyme conjugate in enzyme conjugate buffer (PBST buffer supplemented with 2% polyvinyl pyrrolidone PVP-40 and 0.2% ovalbumin) and incubated overnight at 4°C or 3 to 4 hours at 37°C. Then the plate was washed again with PBST buffer, and each well was filled with 100 µl of 1 mg/ml phosphatase substrate (Sigma 104-105, Sigma Chemical Co.) in the same substrate buffer used for NPTII assays. Absorbance at 405 nm wavelength was recorded after one hour or more of incubation at room temperature. Absorbance values which were at least twice as high as that of healthy untransformed papaya controls were regarded as positive reactions.

CHARACTERIZATION OF R_1 TRANSGENIC PROGENIES

The R_1 progeny were obtained from R_0 plants that were self-pollinated or crossed with untransformed 'Kapoho', 'Sunset' or 'Waimanalo' cultivars in winter and spring of 1992 and 1993. The R_1 seeds from lines 39-1, 39-3, 39-4, 49-2, 55-1, and 60-3 were soaked in 1 M KNO_3 solution for 30 minutes to improve germination (Nagao and Furutani 1986) and sown in vermiculite. In 2 to 3 weeks, seedlings at the full cotyledon stage were transplanted into 3-inch peat pots containing the artificial soil mixture previously described. Two to 5 weeks after transplanting, the seedlings were assayed for GUS, NPTII and CP expression as described above. Six to 8 weeks after transplanting (10 to 30 cm in height), the seedlings were mechanically inoculated in the greenhouse with a severe strain of Hawaiian PRV obtained from infected papaya leaves growing at the Magoon Facility of the University of Hawaii. Fresh virus extract was prepared by grinding one part PRV-infected leaves in two parts (weight/volume) of 0.1 M phosphate buffer pH 7.0 (250 ml of 0.1 M K_2HPO_4 was mixed with 170 ml of 0.1 M KH_2PO_4 to obtain 0.1 M phosphate buffer pH 7.0) with a mortar and pestle as described by Zee (1985). A small amount of carborundum (320 grit), approximately 1/100 (weight/volume), was mixed thoroughly into the extract, and the three youngest fully expanded leaves of R_1 seedlings were inoculated by gently

rubbing the leaf surface with a small pestle dipped into the extract. Five minutes after inoculation, the leaves were rinsed with water to prevent salt damage. The plants were observed for symptom development, and plants that did not develop symptoms were re-inoculated within 2 to 4 weeks. ELISA tests with polyclonal antibody were conducted to confirm symptom observations.

FIELD EXPERIMENT FOR R_0 PLANTS

Previous tests in the greenhouse had shown that R_0 plants of line 55-1 were resistant upon inoculation with Hawaiian isolate of PRV (Fitch et al. 1992). In order to determine how well the coat protein mediated resistance behaved under continuous exposure to PRV inoculum under field conditions, an experiment was installed at the University of Hawaii Waimanalo Experimental Station. Waimanalo is located on the windward side of the island of Oahu at 15 m elevation and has a silty clay soil and annual rainfall of 76 cm. Field Z-I-1 was prepared with 3-m spacing between rows and 2-m spacing between plants in each row. The papaya genotypes that were tested consisted of coat protein-expressing (CP+) female transgenic 'Sunset' line 55-1, female transgenic 'Sunset' line 62-1 which lacked the coat protein gene (CP-) and acted as control for the CP+ plants, and non-transgenic 'Sunset' seedlings used as control for

the transformed plants. The field was also planted with border rows consisting of 'Waimanalo' papaya cultivar. Non-transgenic seedlings were planted on 11 March 1992, and cloned transgenic plants were planted from 11 March 1992 to 1 July 1992, as they became available from tissue culture. The experiment was set up as a split plot design with ten replicates (Figure 1). The main plots consisted of inoculation methods, i.e., manual inoculation versus aphid inoculation, and the subplots consisted of the three papaya genotypes that were compared. Half of the main plots were not artificially inoculated, but were left untouched to test the response of the different papaya genotypes to inoculation by natural populations of aphid vectors. The other mainplots and border rows were manually inoculated on 8 July 1992, and symptomless plants were re-inoculated on 28 July 1992 using the method described previously.

Symptom evaluations were conducted on 11 November 1992, 9 February 1993, 13 April 1993, 8 September 1993 and 3 January 1994. Disease symptoms recorded were leaf mosaic, leaf distortion, petiole lesions, stem lesions, fruit ringspots and fruit distortion. Symptom severity was scaled numerically from 1 to 4, with 1=no symptoms, 2=mild, 3=moderate, and 4=severe. The mean value of all symptom ratings for each plant was used to calculate the overall response of a particular papaya genotype to PRV infection (Zee 1985). Tree vigor, measured as trunk diameter at 45 cm

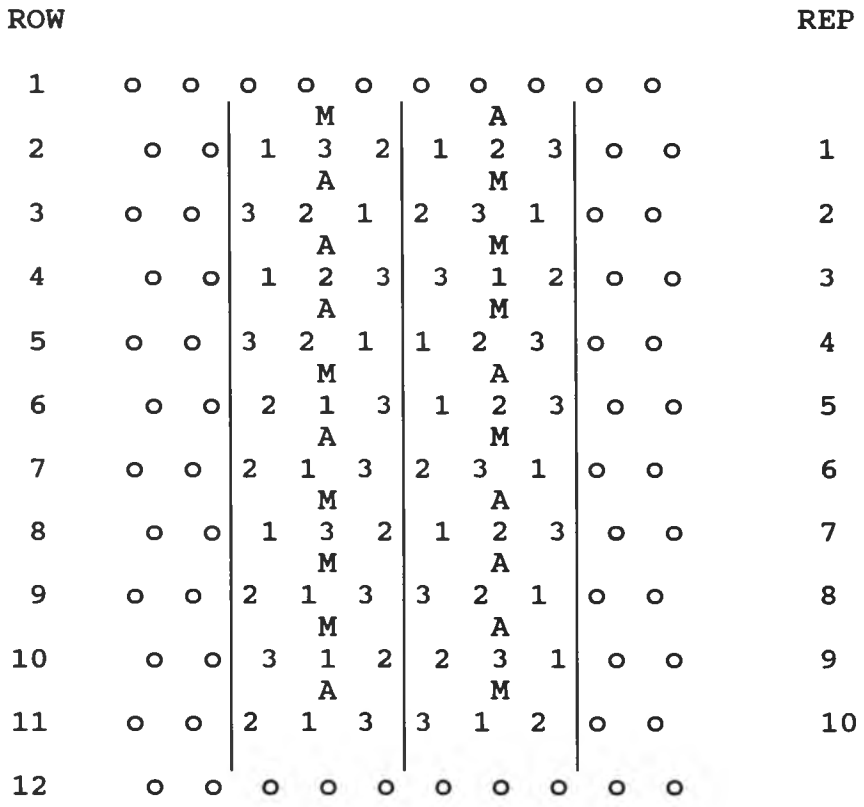

Figure 1. Layout of R_0 transgenic papaya field Z-I-1 at University of Hawaii Waimanalo Station.
A=aphid inoculation (natural vector populations).
M=manual inoculation with PRV-HA (7/8 and 7/28/1992).
o=border rows ('Waimanalo', untransformed seedlings).
1=Cp+ transgenic 'Sunset' line 55-1 (female).
2=Cp- transgenic 'Sunset' line 62-1 (female).
3=Untransformed 'Sunset' seedling.

above ground, was also evaluated. ELISA employing polyclonal antibody (provided by Dr. J. Hu, Department of Plant Pathology, University of Hawaii) was used to monitor PRV development in the field as described above, except that the dilution of antibody-enzyme conjugate used was 1/4000.

Flowering plants of line 55-1 were pollinated with 'Sunset' or 'Kapoho' pollens beginning in September 1992. The fruits were harvested beginning in late January 1993 and evaluated for quality by measuring the percentage of total soluble solids, and their seeds were collected. Open-pollinated fruits of line 55-1 plants were also harvested from time to time for quality evaluation. Other open-pollinated fruits from trees of transgenic phenotypes were removed from the field and disposed of by autoclaving.

FIELD EXPERIMENT FOR R_1 PLANTS

In conjunction with greenhouse testing, R_1 progeny from selected lines were also planted in the field to evaluate their resistance when inoculated by natural aphid vectors. Field Z-I-2 adjacent to the field used for R_0 plants was prepared as described previously for the R_0 plants.

A total of 82 R_1 plants were planted into the field in the first planting on 8 December 1992. Forty-five R_1 plants of CP+ line 39-1, 15 R_1 plants of CP+ line 55-1, 20 R_1 plants of CP+ line 60-3, and 2 plants of untransformed

'Kapoho' cultivar were planted. The 45 R_1 plants of line 39-1 consisted of 40 CP+ plants and 5 CP- plants, originating from seeds of an unbagged self-pollination of an R_0 plant. These seeds and those producing the two 'Kapoho' plants were planted in the greenhouse on 8 August 1992 and PRV-inoculated either on 8 October 1992 or 22 October 1992, and they already showed PRV symptoms in December 1992 prior to transplanting into the field. The 15 R_1 plants of line 55-1 were all CP+ plants originating from crosses with nontransgenic 'Sunset' or 'Waimanalo' cultivars. These seedlings were planted in the greenhouse on 6 April 1992, and PRV-inoculated on 1 July and again on 28 July 1992, but remained symptomless prior to transplanting into the field. The 20 R_1 plants of line 60-3 consisted of 15 symptomless CP+ plants and 5 infected CP- plants, originating from open-pollinated line 60-3. These plants were planted on 19 May 1992, and PRV-inoculated on 7 September 1992 and again on 8 October 1992.

The second set of plantings, as an extension of the first, was done on 29 January 1993 in the same field. Twenty- six R_1 plants of CP+ line 39-1, 26 R_1 plants of CP+ line 55-1, 8 CP+ R_1 plants of line 55-1 left over from the 8 December 1992 planting, and 8 CP+ R_1 plants of line 60-3 left over from the same earlier planting, making up a total of 68 plants, were planted into the field. The 26 R_1 plants of line 39-1 consisted of 20 CP+ and 6 CP- plants.

They were planted in the greenhouse on 21 December 1992. The 26 R_1 plants of line 55-1 consisted of 21 CP+ and 5 CP- plants, originating from a cross made with 'Kapoho' cultivar. They were planted in the greenhouse on 21 October 1992. Seven of the 8 CP+ R_1 plants of line 55-1 left over from planting on 8 December 1992 were already PRV-infected in the greenhouse. The 8 CP+ R_1 plants of line 60-3 were already PRV infected in the greenhouse prior to transplanting into the field.

The third set of plantings was mostly to replace R_1 plants of open-flowered selfed line 39-1. Seventeen CP+ and thirty-three CP- R_1 progeny of selfed line 60-3, planted in the greenhouse on 22 February 1993, were moved into the field on 20 May 1993.

From time to time, mostly at one week intervals, PRV symptom development was recorded in the field. Three to four months after field planting, the plants began to produce flowers. Selected trees were either selfed or crossed with pollen of untransformed 'Sunset' and 'Kapoho' plants to obtain R_2 seeds. Unused hermaphrodite flowers were picked off the trees to prevent transgenic pollen from moving out of the field. Later, open-pollinated fruits from female trees were also collected and disposed of by autoclaving to comply with regulations for transgenic organisms set forth by the USDA.

The R_1 plants were evaluated for PRV symptoms on 14 May

1993, 17 August 1993 and 3 January 1994 as described for R_0 plants. PRV ELISA tests were conducted with polyclonal antibody to confirm the assessment based on visual symptoms.

In order to determine whether infection of CP+ R_1 55-1 plants was caused by a new strain of PRV in Hawaii, bioassays were conducted on 8 September 1993 using inocula from leaves of 9 out of 12 of the infected CP+ R_1 55-1 plants in the field. Each inoculum source was used to inoculate 2 CP+ R_1 55-1 seedlings, 2 CP- R_1 55-1 seedlings, and 1 'Sunset' seedling. The leaves of the test plants were assayed by ELISA to determine virus titer prior to inoculation. Symptom expression was recorded up to 5 months post inoculation.

RESULTS

CHARACTERIZATION OF R_0 TRANSGENIC PAPAYA PLANTS

Most R_0 plants appeared to be normal in morphology and growth characteristics under greenhouse conditions, except for several plants which showed some abnormalities. Plants flowered about 4 months after planting, and their sex phenotype could be determined.

One of 13 plants in the pistillate line 62-1 showed altered leaf and flower morphology. The leaf tip was rather blunt and not sharply pointed, as is characteristic of the normal papaya. It also had abnormal flowers, with narrow, strap-like petals, a capitate stigma instead of the usual lobed stigma, and precocious anthesis (Figure 2). This plant had very poor fertility, and no viable seed was obtained from controlled pollination.

Hermaphrodite plants in line 39-1 showed a marked reduction in pollen fertility as examined with acetocarmine stain. They had an average of 15% or less stainable pollen, while healthy untransformed papaya pollen from the field had pollen stainabilities ranging from 85 to 95%. Interestingly, line 39-1 plants pollinated by untransformed 'Sunset' or 'Kapoho' plants produced substantial amounts of seeds, indicating good egg fertility. Some other hermaphrodite lines also showed reduced pollen stainability, including

Figure 2. Somaclonal variant in R_0 transgenic 'Sunset' line 62-1. Top row: abnormal floral phenotype with narrow, gaping petals and capitate stigma. Bottom row: normal papaya flower.

lines 39-3, 39-4, 44-1, AISAI-ID 1-132, AISAI-ID 4-2 and AISAI-ID 4-4 (Table 2).

Pistillate line 19-1 appeared to be tetraploid. Plants in this line had larger, stiffer, thicker, and darker green leaves, with shorter internodes, stocky flowers and melon-like globose fruits (Figure 3), characteristics of tetraploid papayas as described earlier (Hofmeyr and Elden 1942, Singh 1955). Chromosome counts confirmed the tetraploid nature (2n=36) of the plants in this line. Fertility in this line was greatly reduced, and crossing with normal solo papayas only gave a few viable seeds (Figure 4) or produced parthenocarpic fruits. Thus far, only 5 R_1 seedlings have been produced from this line.

Hermaphrodite line 57-1, as well as three hermaphrodite and one pistillate papaya lines that resulted from *Agrobacterium* transformation, showed growth characteristics similar to the tetraploid papayas described above. They were highly sterile as determined by pollen stainability (Table 2). The four hermaphrodite plants produced small elongated fruit with no viable seeds, while the one pistillate plant produce only a few plump seeds.

Under greenhouse conditions, average fruit weight per line ranged from 200.1 to 563.1 g, and average sugar content (TSS) per line ranged from 9.0 to 16.3% (Table 3). Except for lines 19-1, 39-1, 39-3, 39-4, 57-1 and the *Agrobacterium*-transformed plants, most fruit from the R_0

Table 2. Pollen stainability of R_0 transgenic plants

Line	Cultivar	No. of trees	Pollen stainability (%)
39-1	Kapoho	3	15
39-3	Kapoho	2	22
39-4	Kapoho	1	20
44-1	Kapoho	1	35
AISAI-ID 1-132	Kapoho	1	3
AISAI-ID 4-2	Kapoho	1	4
AISAI-ID 4-4	Kapoho	1	3
33-2	Sunset	1	84
50-2	Sunset	1	79
54-1	Sunset	1	81
57-1	Sunset	1	2
60-3	Sunset	6	78
60-4	Sunset	1	94
62-5	Sunset	1	90
63-1	Sunset	1	12
Untransformed	Kapoho	3	87
Untransformed	Sunset	4	95
Untransformed	Waimanalo	3	85

Figure 3. Somaclonal variation due to tetraploidy. Left: normal floral and fruit morphology in pistillate 'Kapoho' papaya. Right: stubby flowers and pumpkin-shaped fruit in tetraploid pistillate 'Kapoho' R_0 line 19-1.

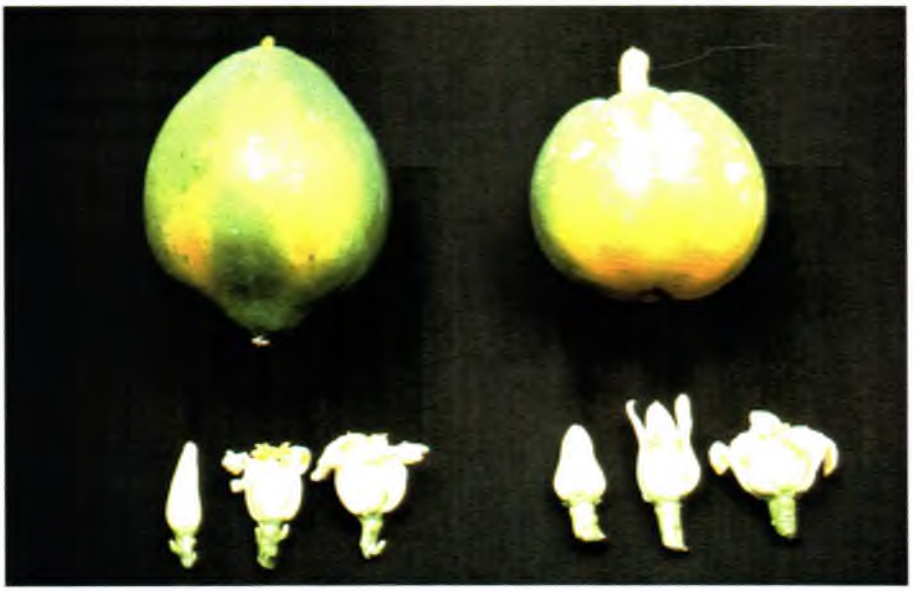

Figure 4. Poor seed set indicating reduced fertility in tetraploid pistillate 'Kapoho' R_0 line 19-1.


GII A9-1-4
x GII 60-3-9
4/16/91

Table 3. Fruit evaluation of R_0 plants grown in Magoon greenhouse

Line	No. of fruit	Weight(g)[a]	TSS(%)[a]
19-1	42	227.0 (120-329)	13.8 (9.5-17.9)
39-1	33	200.1 (50-355.1)	15.0 (6.1-19.4)
39-3	33	225.1 (89.9-372.4)	14.7 (11.9-17.2)
39-4	15	228.7 (85.9-347.6)	15.7 (12.3-17.0)
44-1	2	216.6 (177.6-255.5)	15.1 (12.0-17.9)
AISAI-ID 1-132	1	236.4	16.1 (16.0-16.2)
AISAI-ID 1-137	5	203.1 (155.1-256.6)	12.5 (8.9-15.2)
AISAI-ID 4-2	ne[b]		
AISAI-ID 4-4	7	238.4 (134.7-333.5)	12.9 (11.6-14.3)
33-2	11	354.7 (129.5-558.8)	12.9 (10.2-15.4)
46-1	11	396.0 (109.3-831.0)	17.1 (5.1-19.1)
49-2	42	442.2 (110.5-707.4)	11.3 (6.4-14.6)
50-2	8	404.8 (287.9-576.5)	16.3 (14.5-17.5)
54-1	15	304.5 (217.9-418.9)	13.6 (10.7-18.0)
55-1	113	563.1 (120.2-882.5)	10.6 (6.3-19.2)
57-1	ne[b]		
60-3	68	278.5 (30.3-605.7)	12.8 (7.3-18.6)
60-4	ne[b]		
62-1	42	466.6 (110.9-755.8)	9.0 (4.7-16.9)
62-2	ne[b]		
62-5	17	268.0 (117.4-395.1)	10.3 (7.0-13.2)
63-1	1	312.5	14.8 (14.6-15.0)

[a]Average values with the range in parentheses.
[b]No fruit examined.

plants were normal in appearance and seed production. Pistillate line 19-1 produced only a few seeds per fruit, even though the flowers were heavily pollinated with normal viable pollen. Pistillate line AISAI-ID 1-137 behaved like line 19-1, producing melon-like fruit with only a few viable-looking seeds inside when heavily pollinated with fertile pollen, whereas tetraploid hermaphrodite lines, including the other *Agrobacterium*-transformed plants and line 57-1, produced small elongate fruit without any viable seeds.

Line 39-1 usually produced small fruits with a few seeds when selfed, but quite a large amount of seeds were produced when the flower was outcrossed with fertile pollen. Hermaphrodite lines 39-3 and 39-4 produced carpellodic fruit in the winter months, and behaved similarly to line 39-1 with respect to fertility.

Leaf tissues assayed for GUS expression showed that 13 out of 22 lines tested were GUS positive (Table 4). However, there was a wide range of expression levels in the tissues examined. Lines 39-1, 39-3, 39-4, 55-1, AISAI-ID 1-137, AISAI-ID 4-2, and AISAI-ID 4-4 consistently displayed dark blue color development at the cut edges of leaf tissues within 1 hour. Lines 19-1, 44-1, 49-2, 60-3, 60-4, and AISAI-ID 1-132 showed very mild to moderate levels of GUS expression at the leaf margins or spots in mid-lamina. With the latter set of lines, it was necessary to decolorize the

Table 4. Expression of transgenes in R_0 transgenic plants as determined by X-gluc (GUS) and ELISA (NPTII and CP) assays

Line	GUS	NPTII (A_{405nm})	CP (A_{405nm})
19-1	very weak	weak (0.06-0.47)	+ (0.03-0.94)
39-1	strong	strong (0.28-0.81)	? (0.00-0.03)
39-3	strong	strong (0.76-1.18)	+ (0.07-1.05)
39-4	strong	strong (0.98-1.19)	+ (0.03-0.65)
44-1	weak	moderate (0.42-0.51)	- (-0.01-0.01)
AISAI-ID 1-132	weak?	weak (0.12-0.41)	- (0.00-0.02)
AISAI-ID 1-137	strong	strong (0.90-1.17)	- (0.00-0.02)
AISAI-ID 4-2	strong	strong (0.91-1.19)	- (0.16-0.36)[x]
AISAI-ID 4-4	strong	strong (0.92-1.20)	- (0.01-0.02)
33-2	negative	weak (0.14-0.19)	- (0.00-0.01)
46-1	negative	weak? (0.03-0.12)	- (-0.01-0.02)
49-2	moderate	weak? (0.04-0.28)	? (0.00-0.03)
50-2	negative	strong (0.60-0.81)	+ (0.02-0.08)
54-1	negative	weak? (0.04-0.16)	- (0.00-0.02)
55-1	strong	strong (0.61-1.21)	+ (0.05-0.58)
57-1	negative	strong (0.73-0.91)	+ (0.11-0.79)
60-3	weak?	weak? (0.00-0.29)	+ (0.03-0.62)
60-4	weak	moderate (0.38-0.42)	- (0.00-0.01)
62-1	negative	strong (0.28-0.91)	- (0.00-0.02)
62-5	negative	strong (0.52-0.71)	- (0.00-0.01)
63-1	negative	weak? (0.05-0.06)	+ (0.20-0.88)
Untransformed	negative	negative (0.00-0.04)	- (-0.01-0.02)
PRV infected	negative	negative (0.00-0.03)	+ (0.05-0.91)
NPTII enzyme standard 100 pg/ml		(0.05-0.24)	
NPTII enzyme standard 750 pg/ml		(0.91-1.25)	

[x]Plant spontaneously developed PRV symptoms after the assay.

leaf tissues with ethanol several times in order to visualize GUS expression clearly. Lines 19-1, 60-3, and AISAI-ID 1-132 showed GUS expression inconsistently in several assays, and therefore one assay was not enough to confidently determine GUS expression in these lines. Interestingly, R_1 seedlings produced from these lines, showed consistent medium to strong GUS expression when assayed about 1 to 2 months after germination.

When assayed for NPTII expression, all lines showed positive reactions, although the level of expression varied within and between lines (Table 4). Positive ELISA readings ranged mostly from 0.2 to 1.2 after 30 to 40 minutes of incubation at 37°C, with yield ranging from 3 to 28 ng NPTII/mg of total protein. However, lines 19-1, AISAI-ID 1-132, 33-2, 46-1, 49-2, 54-1, 60-3, 63-1 expressed NPTII weakly, with most of them showing variation that overlapped that of the negative controls. Plants in line 60-3 occasionally did not express NPTII at all.

Expression of CP as assayed by ELISA with monoclonal antibody also showed a wide range of variation (Table 4). ELISA readings for CP+ plants were usually more than twice as high as those of CP- healthy untransformed control plants, and in some plants were occasionally as high as those of PRV- infected control plants. However, plants in CP+ lines 39-1 and 49-2 sometimes failed to express CP and had lower absorbance reading than negative controls. Ten out

of 22 lines were identified as CP+ lines after repeated tests with ELISA (Table 4), which substantiated and extended results obtained previously by Fitch (1991).

CHARACTERIZATION OF R_1 TRANSGENIC PROGENIES

The R_1 seedlings tested for transgene expression were from lines 39-1, 39-3, 39-4, 49-2, 55-1, 60-3, and 62-1. The R_1 progenies of lines 39-1 and 60-3 were produced by self-pollination, while R_1 progenies from lines 39-3, 39-4, 49-2, 55-1, and 62-1 derived from crosses of these lines with nontransgenic 'Kapoho', 'Sunset', or 'Waimanalo' papaya cultivars.

Initial testing with R_1 seeds of line 55-1 revealed that GUS was expressed in seeds (Figure 5), which made it easy to score transgene segregation. However NPTII and CP expression could not be detected in the seeds. Therefore, further tests were performed on seedling leaves. Assays for GUS, NPTII, and CP expressions were conducted simultaneously, and any ambiguities were cleared up by repeating the tests. In most cases, NPTII ELISA was more definitive compared to CP ELISA, in distinguishing positive and negative segregants. Absorbance values for NPTII+ plants were at least 5 times as high as those of NPTII- control plants (Table 5), whereas CP+ plants were sometimes indistinguishable from CP- plants by CP ELISA. The GUS

Figure 5. Segregation (1:1) for GUS expression in backcrossed seeds of R_0 transgenic 'Sunset' line 55-1. The last two columns on the right are seeds of GUS-negative 'Sunset' R_0 line 62-1.

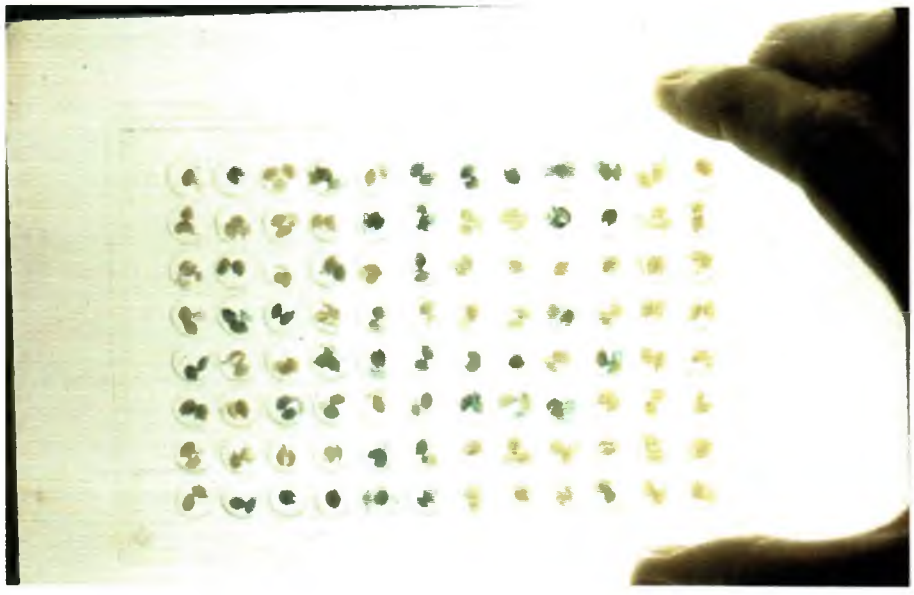

Table 5. Expression of transgenes in R_1 progenies as determined by X-gluc (GUS) and ELISA (NPTII and CP) assays

Line	GUS	NPTII (A_{405nm})	CP (A_{405nm})
39-1	strong	+ (0.32-1.12)	+ (0.02-0.08)
39-3	negative	- (-0.05-0.04)	- (0.00-0.04)
39-4	negative	- (-0.04-0.04)	- (0.00-0.03)
49-2	strong	+ (0.41-0.53)	+ (0.02-0.08)
55-1	strong	+ (0.71-1.26)	+ (0.08-1.12)
60-3	strong	- (-0.03-0.05)	+ (0.02-0.25)
62-1	negative	+ (0.21-1.21)	- (0.00-0.02)
untransformed	negative	- (-0.02-0.04)	- (0.00-0.02)
PRV infected	negative	- (-0.01-0.04)	+ (0.05-0.92)

assay, on the other hand, was mostly obvious, although in some cases doubtful results were obtained, which were later resolved by repeating the assays.

The R_1 seedlings of line 62-1 expressed only NPTII (Table 5), while R_1 seedlings of lines 39-1, 49-2, and 55-1 expressed GUS, CP, and NPTII genes concomitantly as expected, since the three transgenes were tightly linked as one cassette in the plasmid used for papaya transformation (Fitch 1990, 1991).

The R_1 seedlings of line 60-3 expressed GUS and CP, but unexpectedly, no NPTII expression could be detected. The maternal R_0 60-3 plants expressed all three transgenes, though erratic expression of GUS and NPTII was observed from time to time (Table 4). Expression of GUS in the R_1 60-3 seedlings was stronger and more consistent than in the maternal R_0 60-3 plants, but as the R_1 seedlings grew older, GUS expression sometimes became undetectable again.

The R_1 seedlings of lines 39-3 and 39-4 did not express GUS, NPTII or CP at all, which was very unexpected, considering the maternal R_0 plants of these lines expressed GUS, NPTII and CP strongly (Table 4).

The transgenes in lines 49-2, 55-1, and 62-1 segregated in a linked fashion in a 1:1 ratio of positive to negative expression, whereas a 3:1 ratio was observed for line 39-1 (Table 6). However, the expected ratio of 3:1 was not

Table 6. Analysis of transgene segregation in R_1 progenies

Line[a]	Size	GUS (+:-)	NPTII (+:-)	CP (+:-)	X^2	Prob
39-1	90	65:25	65:25	65:25	0.37	0.54
39-3	45	0:45	0:45	0:45	--	--[b]
39-4	39	0:39	0:39	0:39	--	--[b]
49-2	68	33:35	33:35	33:35	0.06	0.81
55-1	394	193:201	193:201	193:201	0.16	0.69
60-3	206	93:113	0:206	93:113	97.92	<0.01[c]
62-1	63	0:63	30:33	0:63	0.14	0.71[d]

[a]Expected ratio of 3:1 for progeny of lines 39-1 and 60-3, 1:1 for lines 39-3, 39-4, 49-2, 55-1, and 62-1.
[b]Chi-square was not calculated.
[c]Chi-square was based on GUS and CP segregation.
[d]Chi-square was based on NPTII segregation.

observed in R_1 seedlings from selfed-pollinated line 60-3, as determined by chi-square analysis.

Resistance to PRV infection was assessed for each of the R_1 progenies in the greenhouse. Response of different R_1 progenies to PRV infection varied from no protection to immunity (Table 7). Susceptible plants usually began to develop stem lesion symptoms, then leaf mottling appeared, and they finally developed leaf distortion.

The R_1 progeny of line 39-1 showed symptoms 2 weeks after manual inoculation, and all plants became infected within 4 weeks post-inoculation. There was no difference between CP+ and CP- plants in terms of viral protection, as both were equally susceptible to PRV. A similar response was observed in the R_1 progeny of line 49-2. CP+ and CP- plants began to show symptoms 3 weeks after inoculation, and by two months after inoculation, all plants had become PRV-infected.

The R_1 progeny of line 60-3 showed a different response to PRV infection. CP- plants began to show symptoms within 3 weeks post-inoculation (Table 7), whereas CP+ plants did not begin to develop symptoms until 5 days later. Most (91%) of the CP- plants had become infected by 30 days post-inoculation, while only 15% of CP+ plants were infected. However, eventually only 16% of the CP+ plants still remained symptomless after 4 months (Table 7). The age of the CP+ seedlings at time of inoculation appeared to be a

Table 7. Response of selected R_1 progenies to PRV inoculation in the greenhouse

Line	CP	No. infected/ No. inoculated	Percent infected	Time of infection[a]
39-1	+	40/40	100	2-4 weeks
39-1	-	12/12	100	2-4 weeks
39-3	-	10/10	100	3-6 weeks
39-4	-	20/20	100	3-6 weeks
49-2	+	33/33	100	3 weeks-2 months
49-2	-	35/35	100	3 weeks-2 months
55-1	+	7/26	27	7 weeks-5 months
55-1	-	36/36	100	2-7 weeks
60-3	+	16/19	84	26 days-4 months
60-3	-	23/23	100	3-7 weeks
62-1	-	53/53	100	15 days-7 weeks

[a]Time post-inoculation.

variable influencing the degree of their PRV resistance, with a greater delay in symptom expression being observed in plants that were inoculated at a later development stage. When inoculated at about 12 weeks after transplanting, 9 out of 24 (37.5%) CP+ plants were infected within 80 days after inoculation, compared to 14 out of 19 (74%) infected in the same time period when inoculated at 6-8 weeks after transplanting.

The R_1 progeny of line 55-1 showed the best protection against PRV infection. Only 8% of the CP+ plants had become infected by the seventh week following inoculation, as determined by ELISA test, though the infected plants still looked symptomless. On the other hand, the CP- plants were all infected within 7 weeks post-inoculation (Table 7). By five months after inoculation, only 27% of the CP+ plants had become infected (Table 7) and displayed very mild vein clearing symptoms. At this point, the non-diseased CP+ plants were transferred from the greenhouse to the field at the University of Hawaii Waimanalo Experimental Station.

The R_1 progeny of line 62-1, which segregated only for NPTII, did not show any protection against PRV infection and behaved just like the untransformed control plants. The plants started to show symptoms within 15 days post-inoculation, and all plants became infected within 7 weeks post-inoculation (Table 7).

The R_1 progeny of lines 39-3 and 39-4, which did not

show any transgene expression, also did not show any protection against PRV infection. They began showing symptoms within 3 weeks post-inoculation, and all were infected by six weeks after inoculation (Table 7).

FIELD TEST OF R_0 PLANTS

The CP+ R_0 line 55-1 performed very well in the field, showing virtual immunity to PRV infection, whereas transformed CP- line 62-1 and untransformed CP- 'Sunset' plants were equally susceptible to viral infection (Tables 8, 9, 10, 11, 12, 13 and 14). The CP+ line 55-1 showed prolific growth during the first few months in the field, in contrast to infected CP- controls (Figures 6 and 7). All manually inoculated CP- controls and border rows of 'Waimanalo' plants showed PRV symptoms within 20 to 30 days after inoculation in the field. Aphid-inoculated CP- controls, which were left for inoculation exclusively by natural aphid vectors, took a longer time before they became infected, but all showed symptoms within 2-4 months after other plants in the experiment were manually inoculated. There was no significant effect of inoculation method on symptom expression on papaya (Table 13), while CP gene expression had a highly significant effect on protection against PRV infection (Tables 13 and 14). Data from CP- line 62-1 and 'Sunset' seedlings were pooled together beginning

Table 8. Evaluation of stem diameter (cm) and PRV symptoms on R_0 plants of lines 55-1 and 62-1, and 'Sunset' seedlings on 11 November 1992, 4 months after first inoculation

Genotype	n	SD[x]	PRV Symptoms			
			LM	LD	PL	SL
55-1	20	8.85a[y]	1.00a	1.00a	1.00a	1.08a
62-1	20	6.65c	3.25b	2.83b	2.77b	2.75b
'Sunset'	20	8.01b	3.13b	2.80b	2.93c	2.65b

[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion.
[y]Values with the same letter are not significantly different.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 9. Evaluation of stem diameter (cm) and PRV symptoms on R_0 plants of lines 55-1 and 62-1, and 'Sunset' seedlings on 9 February 1993, 7 months after first inoculation

Genotype	n	SD[x]	PRV Symptoms					
			LM	LD	PL	SL	FR	FD
55-1	20	12.14a[y]	1.13a	1.03a	1.00a	1.00a	1.00a	1.00a
62-1	20	8.75c	2.55b	2.63b	2.00b	2.08b	2.18b	2.00b
'Sunset'	20	10.26b	2.53b	2.63b	2.38c	2.20b	2.45c	2.55c

[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion, FR=fruit ringspot, FD=fruit distortion.
[y]Values with the same letter are not significantly different. PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 10. Evaluation of stem diameter (cm) and PRV symptoms on R_0 plants of lines 55-1 and 62-1, and 'Sunset' seedlings on 13 April 1993, 9 months after first inoculation

Genotype	n	SD[x]	PRV Symptoms					
			LM	LD	PL	SL	FR	FD
55-1	20	13.28a[y]	1.00a	1.00a	1.00a	1.00a	1.00a	1.00a
62-1	20	9.05c	2.58b	2.85b	2.35b	2.38c	2.20b	2.00b
'Sunset'	20	10.41b	2.55b	2.83b	2.35b	2.13b	2.58c	2.48c

[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion, FR=fruit ringspot, FD=fruit distortion.
[y]Values with the same letter are not significantly different. PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 11. Evaluation of stem diameter (cm) and PRV symptoms on R_0 plants of lines 55-1 and 62-1, and 'Sunset' seedlings on 8 September 1993, 14 months after first inoculation

Genotype	n	SD[x]	PRV Symptoms					
			LM	LD	PL	SL	FR	FD
55-1	20	14.49a[y]	1.00a	1.08a	1.00a	1.00a	1.00a	1.00a
Control[z]	21	8.87b	2.79b	2.71b	2.57b	2.26b	2.08b	2.28b

[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion, FR=fruit ringspot, FD=fruit distortion.
[y]Values with the same letter are not significantly different.
[z]Control values were pooled data from line 62-1 and 'Sunset' seedlings.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 12. Evaluation of stem diameter (cm) and PRV symptoms on R_0 plants of lines 55-1 and 62-1, and 'Sunset' seedlings on 3 January 1994, 18 months after first inoculation

Genotype	n	SD[x]	PRV Symptoms		
			LM	LD	PL
55-1	18	14.69a[y]	1.00a	1.00a	1.00a
Control[z]	14	9.30b	3.29b	2.71b	2.79b

[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion.
[y]Values with the same letter are not significantly different.
[z]Control values were pooled data from line 62-1 and 'Sunset' seedlings.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 13. Effect of inoculation method (manual vs. aphid vector) and papaya genotype [R_0 transgenic CP+ (55-1), R_0 transgenic CP- control (62-1), and CP- control 'Sunset' seedling] on PRV symptom expression

	Date of PRV Symptom Evaluation				
	11/11/92[a]	2/9/93[b]	4/13/93[b]	9/8/93[b]	1/3/94[c]
Inoculation method[d]	2.27:2.26ns	1.86:1.95ns	1.97:1.95ns	1.76:1.72ns	1.96:1.73ns
Papaya genotype	**	**	**	**	**
55-1 vs Controls	1.02:2.89**	1.03:2.35**	1.00:2.44**	1.01:2.45**	1.00:2.93**
62-1 vs 'Sunset'	2.90:2.88ns	2.24:2.45*	2.39:2.48ns	---[e]	---[e]

[a]Average of PRV ratings for leaf mosaic, leaf distortion, petiole lesion, and stem lesion.
[b]Same as above, but including also ratings for fruit ringspot and fruit distortion.
[c]Average of PRV ratings for leaf mosaic, leaf distortion, and petiole lesion.
[d]Manual vs. aphid inoculation.
[e]PRV ratings for 62-1 and 'Sunset' seedlings were pooled to obtain control ratings.
PRV rating scale: 1=no symptoms, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.
ns = not significant.
* = significant ($0.05 > P > 0.01$).
** = highly significant ($P < 0.01$).

Table 14. Effect of CP gene expression in R_0 transgenic plants on susceptibility to PRV infection (measured by ELISA) and on stem diameter

Evaluation date and genotype of papaya	ELISA range A_{405nm}	Stem diameter (cm at 45 cm height)	
NOV. 11, 1992			
Transgenic (CP+)	0.010 - 0.017	8.85	
Control (CP-)[a]	0.681 - 1.914	7.33	**
FEB. 9, 1993			
Transgenic (CP+)	0.020 - 0.084	12.14	
Control (CP-)	0.868 - 1.891	9.55	**
APR. 13, 1993			
Transgenic (CP+)	0.000 - 0.005	13.28	
Control (CP-)	0.157 - 2.138	9.73	**
SEP. 8, 1993			
Transgenic (CP+)	0.000 - 0.014	14.49	
Control (CP-)	0.387 - 0.993	8.87	**
JAN. 3, 1994			
Transgenic (CP+)	0.000 - 0.061	14.69	
Control (CP-)	0.167 - 2.088	9.30	**

[a]Data from line 62-1 and 'Sunset' seedlings were combined.
**Highly significant ($P < 0.01$).

Figure 6. Vigorous growth of R_0 transgenic CP+ line 55-1 with dark green canopy (middle, yellow tape), as compared to R_0 transgenic CP- line 62-1 (right, orange tape) and untransformed 'Waimanalo' border row plant (left, no tape), 5 months after first inoculation in the field.

Figure 7. View of R_0 transgenic trial in December 1992, 5 months after first inoculation (top), and in May 1993, 10 months after first inoculation (bottom). Dark green canopies are of transgenic CP+ line 55-1, in contrast to the chlorotic canopies of transgenic CP- line 62-1 and 'Sunset' seedlings.

with 8 September 1993 evaluation, since several plants of each genotype had died due to the combined effects of PRV and root rot fungi since late April 1993. At eighteen months after the first manual inoculation, still none of the CP+ line 55-1 showed PRV symptoms, whereas CP- controls showed moderate symptoms (Table 13). ELISA tests confirmed the resistant character of CP+ line 55-1 (Table 14); CP+ line 55-1 had ELISA readings ranging from 0.000 to 0.084, in contrast to CP- controls, which had ELISA readings ranging from 0.157 to 2.138.

Expression of the CP gene had significant effects on characters other than PRV resistance. There was a highly significant effect of CP gene expression on tree vigor, as shown by stem diameter measured at 45 cm above ground level (Table 14). Fruit quality evaluation also showed that CP+ line 55-1 was superior to CP- control plants (Table 15). The mean total soluble solids (TSS) in fruit of line 55-1 was about 13%, which was significantly higher than the best fruit collected from the control line 62-1. Fertility of the CP+ line 55-1 was also normal as indicated by seed production in the fruit.

FIELD EVALUATION OF R_1 PROGENIES

Observations in the field showed that R_1 progeny of line 55-1 were quite resistant to PRV and had the best

Table 15. Mean fruit weights and total soluble solids (TSS) of R_0 transgenic plants grown at Waimanalo field

Genotype	n	Weight in grams (range)	Percent TSS (range)
55-1 pollinated	47	924.7(251.8-1231.4)a[x]	13.1(10.1-15.4)a
55-1 open poll.	18	636.1(439.8-1073.7)b	12.7(9.6-15.4)a
55-1 collective[y]	65	844.8(251.8-1231.4)a	13.0(9.6-15.4)a
62-1 open poll.	6	518.1(409.0-706.0)b	11.3(10.2-12.8)b

[x]Values with the same letter are not significantly different.
[y]Fruit data of line 55-1 were pooled for manually pollinated and open pollinated fruit, and compared with line 62-1 fruit in a separate test.

performance of all the transgenic lines tested, though several CP+ plants did become PRV-infected under field conditions (Tables 16, 17, 18 and 19). Of the CP+ R_1 55-1 plants in the first (12/8/1992) and second (1/29/1993) plantings, all of which had been inoculated previously (7/8 and 7/28/1992) in the greenhouse, 7 plants were infected before transplanting to the field, and 5 more plants expressed PRV symptoms within 2 to 5 months after transplanting to the field (Table 20). In total, 12 out of 23 CP+ R_1 55-1 plants (52%) became infected when the plants were inoculated in the greenhouse prior to transplanting to the field. Some of the infected plants showed only very mild vein clearing and leaf mottle, and these occasionally were tested negative for PRV by ELISA, while other CP+ lines, including a few CP+ 55-1 plants, showed severe leaf mottling and distortion (Figure 8, Tables 17 and 18). Interestingly, two of the 12 infected CP+ 55-1 plants recovered completely within 9 to 10 months after the first planting, showing no symptoms and testing negative for PRV by ELISA (Table 18 and 20). These conditions remained unchanged through March 1994.

When R_1 55-1 plants were not inoculated in the greenhouse prior to field transplanting (as in the planting of 29 January 1993), only 2 out of 21 CP+ plants (about 10%) became infected at 3 months after transplanting to the field, whereas 3 out of 5 (60%) CP- plants became infected during the same period (Table 20). At 9 months after

Table 16. Evaluation of stem diameter, PRV symptoms and ELISA values for R_1 transgenic progenies at Waimanalo field on 14 May 1993

Genotype	n	SD[x] (cm)	PRV Symptoms				ELISA (A_{405nm})
			LM	LD	PL	SL	
Kapoho	2	3.60d[y]	3.5	2.5	2.3	2.5	1.945-1.959
I39-1+	40	1.97d	2.9	3.5	2.7	2.3	1.532-2.359
I39-1	5	1.85d	3.0	3.5	2.6	2.1	1.069-2.141
II39-1+	19	3.37bc	2.1	2.1	1.8	1.9	0.003-2.155
II39-1	6	3.08bcd	2.9	3.1	2.5	2.3	0.002-2.120
I60-3+	23	3.84b	3.0	2.9	2.8	2.7	0.006-2.348
I60-3	5	2.10cd	2.5	3.8	2.9	2.3	1.305-2.500
I55-1+	11	5.89a	1.0	1.0	1.0	1.7	0.002-0.016
I55-1+d	12	3.67b	2.5	3.0	2.5	2.4	1.219-2.161
II55-1+	19	3.80b	1.0	1.1	1.0	1.7	0.000-0.009
II55-1+d	2	3.50b	2.0	1.5	2.3	3.0	0.007-1.596
II55-1	5	3.60b	2.5	2.2	1.9	2.2	0.003-2.238

I39-1+=CP+ R_1 39-1 planted on 12/8/1992.
I39-1=CP- R_1 39-1 planted on 12/8/1992.
II39-1+=CP+ R_1 39-1 planted on 1/29/1993.
II39-1=CP- R_1 39-1 planted on 1/29/1993.
I60-3+=CP+ R_1 60-3 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
I60-3=CP- R_1 60-3 planted on 12/8/1992.
I55-1+=CP+ healthy R_1 55-1 planted on 12/8/1992.
I55-1+d=CP+ PRV-infected R_1 55-1 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
II55-1+=CP+ healthy R_1 55-1 planted on 1/29/1993.
II55-1+d=CP+ PRV-infected R_1 55-1 planted on 1/29/1993.
II55-1=CP- R_1 55-1 planted on 1/29/1993.
[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion.
[y]SD values with the same letter are not significantly different.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 17. Evaluation of stem diameter, PRV symptoms and ELISA values for R_1 transgenic progenies at Waimanalo field on 17 August 1993

Genotype	n	SD[x] (cm)	PRV Symptoms				ELISA (A_{405nm})
			LM	LD	PL	SL	
Kapoho	2	4.75def[y]	2.5	2.3	2.0	2.0	1.257-2.070
I39-1+	6	5.58cdef	2.3	2.0	1.8	2.3	0.487-1.681
I39-1	1	9.00abc	2.0	1.0	2.0	2.0	0.759
II39-1+	19	8.75abc	1.8	1.8	1.8	2.2	0.006-1.853
II39-1	6	6.96abc	1.9	2.2	1.9	2.2	0.006-2.174
I60-3+	23	6.94bcd	2.9	2.5	2.5	2.2	0.010-2.280[z]
I60-3	3	3.17f	2.7	3.3	2.5	2.0	1.935-2.076
II60-3+	17	3.72ef	1.2	1.2	1.8	1.6	0.007-1.595
II60-3	26	3.05ef	2.3	1.9	1.9	2.0	0.002-2.334
I55-1+	11	9.14ab	1.0	1.1	1.0	2.0	0.009-0.015
I55-1+d	12	6.94bcde	2.0	1.8	1.3	1.9	0.007-2.018[s]
II55-1+	18	10.60a	1.0	1.0	1.0	1.6	0.009-0.018
II55-1+d	2	8.00abcd	1.3	1.5	1.0	2.0	0.051-1.221
II55-1	5	8.15abcd	2.2	2.2	2.1	2.1	0.007-2.150

I39-1+=CP+ R_1 39-1 planted on 12/8/1992.
I39-1=CP- R_1 39-1 planted on 12/8/1992.
II39-1+=CP+ R_1 39-1 planted on 1/29/1993.
II39-1=CP- R_1 39-1 planted on 1/29/1993.
I60-3+=CP+ R_1 60-3 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
I60-3=CP- R_1 60-3 planted on 12/8/1992.
I55-1+=CP+ healthy R_1 55-1 planted on 12/8/1992.
I55-1+d=CP+ PRV-infected R_1 55-1 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
II55-1+=CP+ healthy R_1 55-1 planted on 1/29/1993.
II55-1+d=CP+ PRV-infected R_1 55-1 planted on 1/29/1993.
II55-1=CP- R_1 55-1 planted on 1/29/1993.
[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion, SL=stem lesion.
[y]SD values with the same letter are not significantly different.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.
[z]One plant of CP+ 60-3 began to recover.
[s]Some I55-1+d plants occasionally recovered.

Table 18. Evaluation of stem diameter, PRV symptoms and ELISA values for R_1 transgenic progenies at Waimanalo field on 3 January 1994

Genotype	n	SD[x] (cm)	PRV Symptoms			ELISA (A_{405nm})
			LM	LD	PL	
Kapoho	2	5.50def[y]	2.3	2.3	1.5	0.818-1.098
I39-1+	6	6.50bcdef	2.9	2.6	2.5	0.808-1.817
I39-1	1	9.50ab	3.0	3.0	2.5	1.433
II39-1+	17	10.00ab	3.1	2.4	2.3	0.271-2.149
II39-1	6	8.17bcd	2.9	2.6	2.3	0.472-1.942
I60-3+	23	7.26bcde	2.8	2.9	2.5	0.007-2.060[z]
I60-3	3	3.50f	3.0	3.5	2.0	1.541-1.700
II60-3+	16	5.81cdef	2.3	2.0	2.1	0.000-1.578
II60-3	23	4.35ef	3.1	3.0	2.6	0.807-2.102
I55-1+	11	9.71ab	1.0	1.0	1.0	0.000-0.061
I55-1+d	12	7.92bcd	2.4	2.4	2.3	0.001-1.811[s]
II55-1+	18	12.83a	1.0	1.0	1.0	0.000-0.041
II55-1+d	2	9.50ab	2.3	2.3	2.0	0.489-1.765
II55-1	5	9.10bc	3.2	2.6	2.7	0.409-1.899

I39-1+=CP+ R_1 39-1 planted on 12/8/1992.
I39-1=CP- R_1 39-1 planted on 12/8/1992.
II39-1+=CP+ R_1 39-1 planted on 1/29/1993.
II39-1=CP- R_1 39-1 planted on 1/29/1993.
I60-3+=CP+ R_1 60-3 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
I60-3=CP- R_1 60-3 planted on 12/8/1992.
I55-1+=CP+ healthy R_1 55-1 planted on 12/8/1992.
I55-1+d=CP+ PRV-infected R_1 55-1 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
II55-1+=CP+ healthy R_1 55-1 planted on 1/29/1993.
II55-1+d=CP+ PRV-infected R_1 55-1 planted on 1/29/1993.
II55-1=CP- R_1 55-1 planted on 1/29/1993.
[x]Abbreviations: SD=stem diameter, LM=leaf mosaic, LD=leaf distortion, PL=petiole lesion.
[y]SD values with the same letter are not significantly different.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.
[z]One plant of CP+ 60-3 recovered.
[s]Two plants of I55-1+d recovered.

Table 19. Average PRV symptom ratings for R_1 transgenic progenies planted at Waimanalo field

Genotype	5/14/1993	8/17/1993	1/3/1994
Kapoho	2.69a[x]	2.19bc	2.00d
I39-1+	2.83a	2.10bc	2.67ab
I39-1	2.80a	1.75cd	2.83a
II39-1+	1.99c	1.92c	2.57abc
II39-1	2.69a	2.04c	2.58abc
I60-3+	2.83a	2.49ab	2.73ab
I60-3	2.88a	2.63a	2.83a
II60-3+	---	1.29e	2.15cd
II60-3	---	2.02c	2.91a
I55-1+	1.18d	1.25e	1.00e
I55-1+d	2.60ab	1.76cd	2.36bcd
II55-1+	1.21d	1.16e	1.00e
II55-1+d	2.19bc	1.44de	2.17cd
II55-1	2.20bc	2.15bc	2.83a

I39-1+=CP+ R_1 39-1 planted on 12/8/1992.
I39-1=CP- R_1 39-1 planted on 12/8/1992.
II39-1+=CP+ R_1 39-1 planted on 1/29/1993.
II39-1=CP- R_1 39-1 planted on 1/29/1993.
I60-3+=CP+ R_1 60-3 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
I60-3=CP- R_1 60-3 planted on 12/8/1992.
I55-1+=CP+ healthy R_1 55-1 planted on 12/8/1992.
I55-1+d=CP+ PRV-infected R_1 55-1 planted on 12/8/1992 (greenhouse healthy) and 1/29/1993 (greenhouse infected).
II55-1+=CP+ healthy R_1 55-1 planted on 1/29/1993.
II55-1+d=CP+ PRV-infected R_1 55-1 planted on 1/29/1993.
II55-1=CP- R_1 55-1 planted on 1/29/1993.
[x]Values with the same letter are not significantly different.
PRV rating scale: 1=no symptom, 2=mild symptoms, 3=moderate symptoms, 4=severe symptoms.

Table 20. Development of PRV disease in terms of cumulative percentage of R_1 transgenic plants infected after transplanting in Waimanalo field

Genotype	n	Months after transplanting										
		0	1	2	3	4	5	6	7	8	9	10
Kapoho	2	100	100	100	100	100	100	100	100	100	100	100
I39-1+	40	100	100	100	100	100	100	100	100	100	100	100
I39-1	5	100	100	100	100	100	100	100	100	100	100	100
II39-1+	19	0	32	37	53	58	68	79	79	95	95	100
II39-1	6	0	33	83	83	83	100	100	100	100	100	100
I60-3+	23	35	44	74	83	87	96	100	100	100	100	96[a]
I60-3	5	100	100	100	100	100	100	100	100	100	100	100
II60-3+	17	0	0	6	12	29	41	59	75[b]	75	75	75
II60-3	26	0	18	52	67	96[c]	96	100	c100	100	100	100
I55-1+	23	30	30	39	44	48	52	52	52	52	48	44[d]
II55-1+	21	0	0	0	10	10	10	10	10	10	10	10
II55-1	5	0	20	40	60	60	60	80	80	80	100	100

I39-1+=CP+ R_1 39-1 planted on 12/8/92 (greenhouse infected).
I39-1=CP- R_1 39-1 planted on 12/8/92 (greenhouse infected).
II39-1+=CP+ R_1 39-1 planted on 1/29/93 (greenhouse healthy).
II39-1=CP- R_1 39-1 planted on 1/29/93 (greenhouse infected).
I60-3+=CP+ R_1 60-3 planted on 12/8/92 (15 plants, greenhouse healthy) and 1/29/93 (8 plants, greenhouse infected).
I60-3=CP- R_1 60-3 planted on 12/8/92 (greenhouse infected).
II60-3+=CP+ R_1 60-3 planted on 5/20/93 (greenhouse healthy).
II60-3=CP- R_1 60-3 planted on 5/20/93 (greenhouse healthy).
I55-1+=CP+ R_1 55-1 planted on 12/8/92 (greenhouse healthy) and 1/29/93 (7 infected, 1 healthy in greenhouse).
II55-1+=CP+ R_1 55-1 planted on 1/29/93 (greenhouse healthy).
II55-1=CP- R_1 55-1 planted on 1/29/93 (greenhouse healthy).
[a] One plant recovered.
[b] One plant died after 7 months in the field.
[c] Seven plants died after 4 months in the field.
[d] Two plants recovered.

Figure 8. Very mild PRV symptom expression in CP+ R_1 55-1 plant (right), as compared to severe PRV symptoms in CP+ R_1 39-1 plant (left).

transplanting, all the CP- plants had become infected, whereas only the same 2 CP+ plants were infected. This situation did not change during the course of the experiment (through March 1994). Furthermore, PRV symptoms on the infected CP+ plants were mild compared to those on the infected CP- plants (Tables 16, 17, 18 and 19), and sometimes they appeared symptomless in the field and tested negative for PRV by ELISA (Figure 9, Tables 16 and 17).

Results from bioassays initiated on 9/8/1993 revealed that infection of CP+ R_1 55-1 seedlings was not due to a new PRV strain in Hawaii. None of the healthy CP+ R_1 55-1 seedlings inoculated with leaf extracts from PRV-infected CP+ R_1 55-1 plants in the field developed symptoms within five months after inoculation (Table 21), while CP- R_1 55-1 and 'Sunset' seedlings began to develop mild to moderate symptoms within 23 days after inoculation. Interestingly, when the PRV titer, as measured by ELISA, was low or undetectable in the inoculum source plant, only part of the CP- bioassay plants became infected upon inoculation. Inoculum derived from a severely infected untransformed papaya was used as a positive control in the bioassays, and it produced more severe symptoms on CP- R_1 55-1 and 'Sunset' seedlings beginning 3 days earlier than the isolates from CP+ R_1 plants. No infections occurred as a result of inoculations with leaf extracts from healthy CP+ R_1 55-1 plants growing in Waimanalo field (Table 21).

Figure 9. Very mild, almost symptomless PRV-expression in CP+ R_1 line 55-1 plant.

Table 21. Evaluation of bioassays using healthy and infected CP+ R_1 55-1 seedlings planted in Waimanalo field as inoculum sources, 5 months after inoculation

Inoculum source[a]	Status[b]	ELISA (A_{405nm})	Infected plants/total plants		
			CP+ R1 55-1	CP- R1 55-1	'Sunset'
1-3	healthy	0.007	0/2	0/2	0/1
1-4	infect	0.644	0/2	2/2	1/1
4-6	healthy	0.006	0/2	0/2	0/1
4-7	infect	0.010	0/2	1/2	0/1
11-1	infect	0.005	0/2	1/2	1/1
11-2	infect	0.965	0/2	2/2	1/1
11-3	infect	0.524	0/2	1/2	1/1
11-4	infect	0.896	0/2	2/2	1/1
13-7	infect	0.818	0/2	2/2	1/1
13-8	infect	0.854	0/2	2/2	1/1
13-9	infect	0.902	0/2	2/2	1/1
Magoon	infect	1.516	0/2	3/3	1/1

[a]Row and tree number of CP+ R1 55-1 plants in the field.
[b]Status of plant with respect to PRV infection: healthy or infected, as determined by symptoms and previous ELISA tests.

Measurements of stem diameter at 45 cm above ground level showed that CP+ R_1 progeny of line 55-1 had the most vigorous growth in the field (Figure 10), especially those plants that were not inoculated prior to field transplanting (Tables 16, 17, 18). Fruit quality of CP+ R_1 55-1 was also the best among the transgenic lines tested, with average fruit weight of 577.3 g and average TSS of 13.3% (Table 22).

The R_1 progenies of line 39-1 failed to show any useful level of protection to PRV infection under field conditions. The greenhouse-inoculated CP+ and CP- plants were equally susceptible to viral infection, and did not recover in the field. However, the CP+ plants that were not manually inoculated prior to field transplanting had significantly milder symptoms at the beginning of the evaluation period, although the protective effect was lost after 6 months in the field (Table 19). The CP+ plants also showed a delay in symptom expression which was not observed when plants were manually inoculated in the greenhouse (Table 20). Among the R_1 seedlings planted without prior manual inoculation, 100% of the CP- plants had become naturally infected within five months after transplanting, whereas the CP+ plants were not completely infected until 10 months after transplanting. The stem diameters of the naturally inoculated CP+ plants were larger, although not significantly different, than those of the CP- plants after 1 year in the field (Table 18).

Symptom expression in R_1 progeny of line 60-3 was mild

Figure 10. More vigorous growth of CP+ R_1 55-1 plants (3 plants in foreground), as compared to severely infected R_1 39-1 plants (5 smaller plants in background).

Table 22. Mean fruit weights and total soluble solids (TSS) of CP+ R_1 transgenic progenies grown at Waimanalo field

Genotype	n	Weight in grams (range)	Percent TSS (range)
55-1	130	577.3 (109.3-1196.2)a[x]	13.3 (6.6-17.6)a
60-3	14	301.6 (73.8-464.5)b	11.3 (4.9-13.8)b
39-1	13	212.8 (33.2-665.6)b	11.1 (7.5-14.0)b

[x]Values with the same letter are not significantly different.

to severe under field conditions, though on the average, the CP+ plants were slightly better (Table 19). Healthy CP+ plants which were inoculated in the greenhouse prior to transplanting to the field developed PRV symptoms within 1 to 6 months in the field (Table 20), whereas all the CP- plants had become infected in the greenhouse prior to transplanting. Interestingly, 1 out of the 23 CP+ infected plants recovered completely after 10 months in the field (Tables 17, 18 and 20), showing no symptoms and testing negative for PRV by ELISA. The CP+ plants also had significantly larger stem diameters than their CP- counterparts, but unequal sample sizes may have biased this comparison (Tables 16, 17, 18). For R_1 progeny of 60-3 which were not inoculated in the greenhouse prior to transplanting, 75% of the CP+ plants developed symptoms within 7 months after planting, with the majority of these (41%) acquiring the disease within 5 months (Table 20). At ten months after transplanting, there were still 4 out of 16 CP+ plants (25%) that remained healthy in the field. The CP- plants, on the contrary, became infected within 6 months after planting (Table 20), with the majority (67%) acquiring the disease within 3 months. The symptom severity on CP+ plants was also milder than that on the CP- plants (Tables 17, 18 and 19).

DISCUSSION

CHARACTERIZATION OF R_0 TRANSGENIC PLANTS

Altered morphology, polyploidy, and reduced fertility, as the result of somaclonal or other kinds of variation, were generated with high frequency by the transformation methodology adopted. Twelve out of 22 R_0 lines evaluated were affected, with most of them showing reduced pollen fertility (Table 2). In one of the lines affected, line 62-1, only 1 out of 13 plants examined showed somaclonal variation, while in other lines, all the plants showed the subnormal phenotypes. These might have been caused by long-term exposure of explants to 2,4-D in tissue culture step before regenerants were produced. Somatic embryo cultures were exposed to 2,4-D in much longer period than zygotic embryo cultures (Fitch and Manshardt 1990, Fitch 1991). Five out of six tetraploid lines evaluated were from somatic embryo cultures, whereas the other 1 line was from zygotic embryo culture.

Transgene expression varied in different lines studied, even when the same gene construct was used for transformation. Different patterns and levels of transgene expression from the same construct and transformation procedure have been reported previously (Nagy et al. 1985, Shirsat et al. 1989, Barnes 1990). In present study,

variation in expression of transgenes was observed for plants within the same line, although the differences were more obvious among different lines. The CP gene was flanked by the GUS and NPTII genes in the plasmid carrying the expression cassette (Fitch 1991, Fitch et al. 1990), however some of the lines that expressed GUS and/or NPTII did not have CP expression, suggesting that fragmentation of the plasmid had occurred during transformation. Foreign genes in plants are usually transcribed in a regulated rather than constitutive manner, and expression is usually tissue specific and subject to environmental influences (Kuhlemeier et al. 1987, Benfey and Chua 1989). Line 60-3 expressed GUS and NPTII erratically, suggesting that the construct was subject to developmental regulation. However, effects of transgene copy number, position of gene insertion into the chromosome, DNA methylation, and presence of trans-acting factors in the vicinity of transgenes might also account for the differences in expression observed (Weising et al. 1988).

FIELD TESTING OF R_0 PLANTS

Previously the CP+ R_0 plants of line 55-1 have been shown to be resistant to PRV infection under greenhouse conditions (Fitch 1992). The resistance also proved to be effective under continuous PRV inoculation by aphid vectors

in the field. Twenty months after the first inoculation in the field, none of the CP+ R_0 plants of line 55-1 showed viral symptoms, in contrast to the CP- plants which developed moderate symptoms (Table 13). As expected, growth of the CP+ R_0 plants, as measured by stem diameter, was more vigorous than that of the controls (Table 14). This might have made the CP+ plants more tolerant of root rot fungi compared to the controls, as shown by the total number of plants left in the field as of January 1994, 18 months after the first inoculation (Table 12). By this date, however, there were many more transformed CP- line 62-1 plants left in the field (13 plants) than 'Sunset' seedlings (1 plant). Transgenic plants in line 55-1 and 62-1 were clonally propagated by tissue culture. It might be possible that the tissue culture procedure had made the transgenic plants more resistant to root rot fungi compared to the control seedlings. Different planting dates might also have caused the difference, since some of the plants in transgenic line 62-1 were plants replanted later in the field replacing earlier plantings that died.

No viral replication was observed in the CP+ R_0 55-1 plants as revealed by the ELISA tests (Table 14), indicating an immune response to PRV infection. However, a single, small branch near the base of one plant showed PRV symptoms in June 1993. Leaves from this branch tested positive for PRV by ELISA with polyclonal antibody, but the main canopy

of the plants remained symptomless and ELISA negative. The infected branch, however, still showed GUS and NPTII expressions. Severe growth stress in the field might have accounted for this phenomenon, as the plant was not growing well, fell down to the ground in September 1993, and finally died due to root rot disease.

No inferior growth characteristics were observed due to the additional metabolic load imposed on the plants by the extra genes, which might have resulted in reduced fitness. The CP+ plants produced good quality fruit with TSS about 13% (Table 15), acceptable for the consumer market.

CHARACTERIZATION OF R_1 PROGENY IN THE GREENHOUSE

GUS was expressed in embryo and endosperm tissues of R_1 seeds in the progenies tested, but NPTII and CP could not be detected in seeds. NPTII was not expressed in seeds, suggesting that it was developmentally regulated or tissue-specific in their expression. CP ELISA tests for seeds gave false positive results, and there was no difference in the absorbance readings between CP+ and CP- seeds. In most cases, all transgenes expressed in R_0 plants were also expressed in R_1 seedling leaves in a consistent fashion, with a few exceptions. For example, we found that R_1 progeny of line 60-3 expressed GUS in seeds and small seedlings, but the expression was erratic in later growth stages. Also, R_1

progeny of line 60-3 never expressed NPTII at any developmental stage, and the R_1 progenies of lines 39-3 and 39-4 failed to express any of the three transgenes demonstrated in the R_0 maternal plants. Developmental regulation or gene inactivation due to methylation might have accounted for the erratic transgene expression in progeny of line 60-3. Loss of transgene expression in seedlings growing without selection on medium lacking kanamycin has been reported previously (Matzke and Matzke 1990), and might also be the cause for the loss of NPTII expression in progeny of line 60-3. Loss of genes during meiotic propagation, or integration of the transgenes into loci essential for gamete or embryo development (Mittelsten Scheid et al. 1991), might be involved in the absence of transgene expression in progenies of line 39-3 and 39-4.

Analysis of R_1 progeny segregation showed that GUS, NPTII and CP transgenes were tightly linked, and most of the transgenes were stably incorporated into the papaya genome. Foreign genes are usually inherited in a Mendelian manner (Budar et al. 1986), and in most of the R_1 papaya progenies tested, a simple Mendelian segregation ratio was observed, indicating that the transgenes were integrated at a single locus (Table 5). However, selfed R_1 progeny of line 60-3, and backcrossed R_1 progeny of line 39-3 and 39-4 showed deviations from the Mendelian trait inheritance (Table 6). All deviations consisted of fewer than the expected number

of transgenic R_1 segregants, or as in line 39-3 and 39-4, their complete absence. Suppression of transgene expression in plants homozygous for transgenes has been reported previously (de Carvalho et al. 1992). High proportion of transgenic progenies with non-Mendelian trait inheritance (lower than expected transmission) have been observed, which correlated with a high copy number of gene insertion (Deroles and Gardner 1988a,b). These might have accounted for the erratic segregation in selfed progeny of line 60-3. Larger progeny sizes from selfed-pollination and backcrosses will be needed to confirm the anomalies in our data. Further studies in molecular genetics will also be needed to determine if the transgenes in R_0 plants giving rise to non-Mendelian ratios in the R_1 generations are integrated at a single locus, either as a single copy or as a cluster of tandem copies, and to possibly elucidate the mechanisms underlying failure of transgene expression in segregating progeny. Knowledge of how transferred genes are inherited in subsequent generations will be of importance if transgenic papaya plants are to have any impact on commercial agriculture.

Inoculation of R_1 progenies with PRV showed that the CP gene afforded different levels of protection in different lines, ranging from no protection to high level resistance (Table 7). The R_1 progenies of lines 39-1 and 49-2 did not show any resistance to PRV infection when manually

inoculated; both CP+ and CP- plants were equally susceptible. In line 60-3, a low percentage of CP+ R_1 plants showed a delay of symptoms or even resistance, and plants inoculated at 13 weeks after transplanting seemed to have a longer disease-free period (8 healthy out of 16 plants at 4 months after inoculation) than plants inoculated at 7 weeks after transplanting (3 healthy out of 19 plants at 4 months after inoculation), a phenomenon observed previously (Fitch et al. 1992). In line 55-1, despite a 27% infection rate, the remainder of R_1 plants were completely resistant to PRV inoculation under greenhouse conditions (Table 7). Occasional inactivation of antibiotic resistance genes has been reported to occur at low frequency (Saul and Potrykus 1990, Mittlesten Scheid et al. 1991), and methylation is one factor causing gene inactivation in transgenic plants (Matzke et al. 1989, Matzke and Matzke 1990). Plants which expressed high level of CP occasionally produced some cells with low or no expression CP (Register and Beachy 1988). In addition, stress conditions in the greenhouse during the course of the experiment might also contribute to breakdown of resistance.

There is no clear reason why different R_1 progenies expressing the CP gene responded differently to PRV inoculation. One possible mechanism might involve the level of CP expression, with PRV resistance being correlated with CP concentration. Other researchers have shown that CP

levels in transgenic plants do not correlate well with the level of protection afforded against viral infection (Stark and Beachy 1989, Quemada et al. 1991, Regner et al. 1992), and in these cases, resistance could only be determined by inoculation of the CP+ plants with challenge virus (Beachy 1993).The level of CP expression in R_1 papaya seedlings within a particular line did not correlate well with individual levels of PRV resistance. However, seedlings of line 55-1 and 60-3, which had higher CP expression than seedlings of line 39-1 and 49-2 (Table 5), were better protected, suggesting a basal level of CP might be needed for viral protection.

Modification of CP gene product, which might result from gene rearrangement, has been shown to affect resistance in transgenic plants (Regner et al. 1992, Lindbo and Dougherty 1992a). In our case, Western blot analysis has shown that line 55-1 expresses a CP molecule that is smaller than that predicted for the CP of PRV HA 5-1, from which the CP gene was derived (Gonsalves personal communication).

Line 55-1 has also been shown to have an RNA banding pattern different from the other CP+ lines (Fitch et al. 1992), suggesting that RNA-mediated protection (Lindbo and Dougherty 1992b) might be involved in high-level PRV resistance.

The position effect, due to differences in the physical locus of gene insertion, probably influences transgene

expression in different transformants carrying the same construct. Position effects have been implicated in differential expression of transgenes (Barnes 1990, Clark et al. 1990), and might also account for the differences observed in PRV resistance in transgenic papaya.

Further study at the molecular level is needed to unravel the mechanisms lying behind the resistance.

FIELD TESTING OF R_1 PROGENIES

Of three CP+ lines tested, R_1 progeny of line 39-1 showed the least protection against PRV infection. CP+ and CP- plants in this line responded to manual PRV inoculation in the greenhouse with equal susceptibility in the field. However, without greenhouse inoculation prior to field transplanting, the CP+ plants showed less severe symptoms than CP- plants during the first few months of the field test, but degenerated to the same level within 7 months (Table 19). Growth, as measured by stem diameter, was also more vigorous for these CP+ plants, suggesting that under mild disease pressure, CP+ expression in line 39-1 provided weak protection against PRV infection.

The R_1 progeny of line 60-3 gave a better response to PRV infection. CP+ plants showed a delay in symptom development and less severe symptoms compared to CP- plants, especially when transplanted in the field without prior

inoculation in the greenhouse (Table 19). At the last observation made 9 months after field transplanting, 23.5% of the CP+ plants (4 out of 17 CP+ plants) remained symptomless. Growth, as indicated by stem diameter, was more vigorous for the CP+ plants (Table 16, 17, 18), suggesting that CP expression in line 60-3 gave a certain level of protection against PRV infection. One infected CP+ plant in this line underwent a spontaneous remission, showing no PRV symptoms and no viral replication as tested with ELISA, by a mechanism as yet unknown.

The R_1 progeny of line 55-1 showed a high level of resistance, if not immunity, in most of the CP+ plants examined in the field. Although half of the CP+ plants became infected in the field if they were manually inoculated in the greenhouse, only a small percentage (9.5%) of the CP+ plants transplanted in the field without prior greenhouse inoculation got infected (Table 20). Severe stress due to manual inoculation and unfavorable growth conditions in the greenhouse might account for the different response. However, hybrid vigor might also be involved, since the manually inoculated R_1 55-1 plants consisted mostly of inbred seedlings derived from a backcross with 'Sunset', while the latter R_1 55-1 were obtained from F_1 hybrid cross with 'Kapoho'. CP+ plants without prior manual inoculation grew faster and more vigorously than the manually inoculated CP+ plants, as shown by the stem

diameter measurements (Table 16, 17, 18). Fruit quality of the CP+ R_1 plants (Table 22), as in the R_0 plants with TSS averaging 13%, was acceptable for commercial cultivars.

The results indicated that protection by the CP was partly compromised when the R_1 plants were manually inoculated. Nevertheless, the overall performance of the infected CP+ R_1 55-1 plants was still better than that of other lines tested (Table 19). Viral replication appeared to be inhibited in infected CP+ 55-1 plants, judging from reduced symptom severity, negative or very low ELISA reading, and even remission of PRV symptoms.

Bioassays on healthy untransformed papaya plants and seedlings of line 55-1, using inocula from leaves of infected CP+ 55-1 plants and healthy CP+ 55-1 plants, confirmed that coat protein-mediated protection interfered with viral multiplication (Table 21). No infection occurred on CP+ 55-1 seedlings in the bioassays, indicating that the breakdown of coat protein-mediated protection in CP+ R_1 55-1 plants inoculated in the greenhouse was not due to selection of a new virulent PRV strain.

There were two infected CP+ R_1 55-1 plants that showed complete remission as was also observed in one of the CP+ R_1 plants of line 60-3. Reversible methylation has been found to play a crucial role in gene activation/inactivation in transgenic tobacco plants (Matzke et al. 1989, Matzke and Matzke 1990) and is possibly responsible for PRV remission

in the transgenic papaya. It is possible that viral infection of CP+ 55-1 plants occurred during a stage when the CP transcription level was low, and a subsequent increase in CP transcription at a later stage might restore the resistance to PRV (Mittelsten Scheid et al. 1991). Further study is needed to elucidate the mechanisms underlying this phenomenon of remission.

SUMMARY

As transgenic papayas had been produced by previous work (Fitch and Manshardt 1990, Fitch et al. 1990, Fitch 1991), the next step to characterize the transgenic plants for transgene expression and to evaluate plant performance in accordance to inheritance of the transgenes were the main objectives in this work.

We had characterized 22 R_0 transgenic lines growing in the greenhouse for transgenes: β-glucuronidase (GUS), neomycin phosphotransferase II (NPTII), and papaya ringspot virus coat protein (CP); and morphological characteristics. Somaclonal and other kinds of variation, including altered morphology, polyploidy, and reduced fertility had been found to affect individual plants in 12 out of 22 transgenic R_0 lines evaluated. Nevertheless, R_1 seeds had been produced from most of the transgenic lines. Transgene expression in each of the R_0 lines was highly different, ranging from no expression to high level of expression. Developmental regulation had also been found to affect the transgene expression.

R_1 progeny obtained from crosses in the greenhouse were also characterized to determine the nature and stability of transgene expression after sexual segregation. Seven different R_1 progeny were tested, with a varying degree of expression. Most of the transgenes segregated according to

simple Mendelian inheritance, with some exceptions which might need further investigation for clarification. R_1 progeny of line 55-1 had been found to perform satisfactorily after viral inoculation, with a high level of resistance under greenhouse conditions, while other were susceptible or had a delayed symptom expression.

Field evaluation for PRV resistance had been conducted for selected R_0 plants. CP+ R_0 line 55-1 performed extraordinarily well in the field, with a high level of resistance, if not immune, as had been previously shown in the greenhouse (Fitch et al. 1992). Tree performance as evaluated by symptom ratings and stem diameter was contrastingly superior in this line compared to the CP- control plants. Line 55-1 was also morphologically normal and produced acceptable fruit for the market.

In order to know the inheritance of the transgenes, particularly the CP gene expression in the field, field evaluation for the R_1 progeny of different CP+ lines was also conducted. However, only R_1 progeny of line 55-1 performed well in the field, with highly resistance characteristics as the maternal R_0 plants, despite some infected trees with minor symptom expression. R_1 progeny of line 60-3 showed a delay and reduced level of symptom expression, while R_1 progeny of line 39-1 did not showed obvious protection against PRV infection. Interestingly, 3 plants of infected CP+ R_1 progeny of line 55-1 and 60-3

recovered completely from viral infection after 9-10 months in the field. This suggested developmental stage of the CP+ plants might affect their performance under disease pressure. Further study on transgene inactivation/activation might be needed in order to elucidate the mechanisms underlying this phenomenon.

Our results had shown that from all 22 R_0 transgenic lines examined, 1 line (line 55-1) was virtually effective to give protection against PRV in papaya. R_2 progeny evaluation of this line is currently underway. Since it had been shown that the 3 transgenes segregating according to Mendelian laws in this line, there is 25% chance to obtain homozygous plants for CP in the R_2 generation. They will serve as maternal plants to produce transgenic papaya plants resistant to PRV disease and solve the major disease problem for papaya production in Hawaii. This is likely to happen, provided USDA regulations and the society in common, has come to a resolution to accept transgenic plant material for consumption in the near future.

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